DOCTOR OF PHILOSOPHY

The expression and role of Migration Stimulating Factor (MSF) in oral tumours

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University of Dundee

DENTAL SCHOOL

THE EXPRESSION AND ROLE OF MIGRATION STIMULATING FACTOR (MSF) IN ORAL TUMOURS

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THE EXPRESSION AND ROLE OF MIGRATION STIMULATING FACTOR (MSF) IN ORAL TUMOURS

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DECLARATION

I declare that I am the author of this thesis and that I have consulted all the references cited. The work of which this thesis is a record has been done by myself and has not previously been accepted for higher degree. This work has been carried out in the Unit of Cell and Molecular Biology, University of Dundee, Dental Hospital and School, under the supervision of Dr Ana M Schor, Dr Ian R Ellis and Dr Sarah J Jones.

Signed……..

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Certificate

I hereby certify that Lateef Aljorani has fulfilled the conditions of ordinance 39 of the University of Dundee and is qualified to submit thesis for the degree of Doctor of Philosophy in Oral Pathology.

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DEDICATION

To

The immortal spirit of my parents
whose presence are always with me
My wonderful and great wife for her
encouragement and unlimited support
My lovely kids Ghada and Mustafa

LIST OF ABBREVIATIONS

Ab	Antibody
ABC	Avidin Biotin Complex
AC	Adenocarcinoma
ACC	Adenoid cystic carcinoma
ADH	Alcohol dehydrogenase
Ang	Angiomyleiomyoma
BcAC	Basal cell adenocarcinoma
Bcl-2	B-cell CLL/lymphoma 2 protein interfering with normal apoptosis
bp	base pair
BSA	Bovine serum albumin
Bv	Blood vessel
CA 125	Cancer antigen 125
CD117	C-kit proto-oncongene
CDKs	cyclin dependent kinases
CDKN2A	cyclin-dependent kinase inhibitor 2A
cFn	cellular fibronectin
CK	Cytokeratins
CM	Conditioned medium
CMB	cell molecular biology
c-myc	Myc protooncogene protein Myelocytomatosis transcription factors
Conc	Concentration
Cox-2	Cyclooxygenase-2
CSF	cerebrospinal fluid
CT	computer tomography
CYFRA 21-1	cytokeratin 19-fragments
DCS	Donor calf serum
DEAE	Diethylaminoethyl cellulose
DFOM	Deferoxamine Mesylate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPX	mountant material
E	Eluted
E7	E7 papillomavirus protein

EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eMSFI	endothelial Migration Stimulating Factor Inhibitor
erbB1	epidermal growth factor receptor
ERK1/2	extracellular signal-regulated Kinase
ET	EGTA Trypsin
EU	endotoxin units
Exd	excretory duct
Exp	Experiment
FAK	focal adhesion kinase
Fb	Fibroblast
FCD	Fetal calf serum
FD	Freeze-dried
FDI	World Dental Federation
FEEGF	histidine residues in HEEGH mutated to phenylalanine (F)
FGF	Fibroblast growth factor
Fig	Figure
Fn	Fibronectin
FNAC	Fine needle aspiration cytology
GBD	Gelatin binding domain
GFAP	glial fibrillary acidic protein
Gy	Gray unit for absorbed dose of ionizing radiation
H_2SO_4	sulfuric acid
HA	Hyaluronic acid
HaCat	keratinocytes cell line
HBD	heparin-binding domain 1
HBBS	Hanks balance salt solution
HCC	human hepatocellular carcinoma cells
HCl	Hydrochloric acid
HEEGH	putative zinc-binding motif
HER	Human epidermal growth factor receptor
HNSCC	Head and Neck squamous cell carcinoma
Hp	Hyperplasia

Hpb	hyperplasia basal layer
Hpsb	hyperplasia suprabasal layer
HPV	Human Papillomavirus
HRP	Horse Radish Peroxidase
HSV	Herpes simplex virus
IARC	International Agency for Research on Cancer
IC	inflammatory cell
ICC	Immunocytochemistry
ICD	intercalated duct
IGD	Isoleucine-glycine-aspartic acid
IGF	Insulin likes growth factor
IHC	Immunohistochemistry
IPTG	Isopropyl-β-D galactopyranoside
IQR	inter-quartile range
iso*DGR*	isoaspartate-glycine-arginine
ITF	invasive tumour front
kDA	Kilo Dalton
Ki-67	cellular marker for proliferation
Kp	Keratin pearl
LPS	lipopolysaccharides
MAB mab	monoclonal antibody
MC	Mucoepidermoid Carcinoma
MCF-7	breast tumour cell line
MDAMB435	breast cancer cell line
MEM	Minimum Essential Medium Eagle
MMPs	Matrix Metalloproteinase
MRI	magnetic resonance imaging
MSF	Migration Stimulating Factor
MSFI	Migration Stimulating Factor Inhibitor
µg/ml	microgram/millilitre
NaCl	sodium chloride
Na HCO_3	sodium hydrogen carbonate
ng/ml	nano gram/millilitre
NGAL	Neutrophil gelatinase-associated lipocalin
NGS	Normal Goat Serum
NM 23	non-metastatic cells
NSG	Normal salivary gland

OD	Optical density
OSCC	Oral squamous cell carcinoma
PA	Pleomorphic adenoma
p21	Cyclin-dependent kinase inhibitor
p53	Tumour suppressor gene
PBS	Phosphate buffering saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PDT	Photodynamic Therapy
pg/ml	pico gram/millilitre
PI3-K	Phosphoinositide 3 kinase
pRb	retinoblastoma protein
PVDF	Polyvinylidene fluoride
Ras	small GTPase of the Ras homolog family
Rb	Retinoblastoma
RGD	Arginine- glycine-aspartic acid
rh	recombinant human
RNA	Ribonucleic acid
rpm	revolutions per minute
RT	Room temperature
SC	Sebaceous carcinoma
SCC	Squamous cell carcinoma
SD	Standard deviation
Sd	Striated duct
SDS-PAGE	sodium dodecyl sulfat polyacrylamide gel electrophoresis
SEER	the Surveillance, Epidemiology and End Results
SF	Serum free
SGC	salivary gland carcinoma
SGT	Salivary gland tumour
TBS	Tris buffer saline
TBST	Tris buffer saline tween
TGF	Transforming growth factor
TGS	Tris Glycine SDS
TMB- T	Tetramethylbenzidine
TNM	Classification of Malignant Tumours
UICC	Union for International Cancer Control
UTR	Untranslated region

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial grotwh factor receptor
WHO	World Health organisation
WT	wild type

ABSTRACT

Migration Stimulating Factor (MSF) is an oncofoetal protein which is constitutively produced by both epithelial and stromal cells during foetal development, not expressed by the majority of their normal adult counterparts, but re-expressed during pathological processes such as cancer and wound healing. Scotland has the highest occurrence of oral cancers in the UK; the incidence is still increasing, but patient survival remains very poor. The expression of MSF in oral tumours has not been previously reported.

The aims of this study were:

- To determine the effects of MSF on the migration of oral tumour cell lines and normal stromal cells in culture (chapter 3),
- To ascertain the possible presence, diagnostic and prognostic value of MSF in oral squamous cell carcinoma (OSCC; chapter 4), and salivary gland tumours (SGT; chapter 5).
- To identify the putative MSF receptors in oral tumour cell lines (chapter 6).

For tissue culture studies, the effects of rhMSF (wild type and mutant proteins) were examined on human cell lines TYS, HSG, Endo 742 and FSF44. These cells were derived from OSCC, SGT, microvascular endothelial cells and skin fibroblasts, respectively. For *ex-vivo* studies, paraffin embedded archival specimens of OSCC and SGT were stained with specific MSF antibodies and the level of staining was assessed by consensus of 2-4 independent observers. The association between MSF expression and patient survival was determined by Kaplan-Meier and log-rank tests.

Results presented in this thesis indicate that TYS and HSG cells secrete bioactive MSF in culture. rhMSF stimulated the migration of these tumour cells. The use of mutant proteins demonstrated marked differences among the cells examined: Five bioactive motifs (4x IGD and 1x HEEGH) were required for MSF bioactivity on TYS and HSG cells, whereas only one of these motifs was required for Endo 742 and two for FSF44. MSF+aa and MSF-aa showed the same migration-stimulating activity, but differ in their interaction with the MSF-inhibitor Neutrophil Gelatinase-Asscciated Lipocalin (NGAL). NGAL was shown to bind to and inhibit MSF+aa, but

not MSF-aa. The bioactivity of MSF+aa and MSF-aa was inhibited by Insulin-like Growth Factor Binding Protein-7 (IGFBP7), MSF-function-neutralising antibody and antibody to the integrin αvβ3. This integrin was identified in the cell membrane material bound to MSF, suggesting that αvβ3 is a receptor for MSF.

Analyses of MSF staining in tissue sections indicated that MSF is expressed by most OSCC and malignant SGT, being heterogeneously present in both carcinoma and stromal cells. In SGT, significantly higher levels of total MSF and MSF-aa were detected in malignant than in benign tumours. In OSCC, high MSF expression in the invasive tumour front (ITF) was significantly associated with poor patient survival. MSF-aa was more informative than total MSF.

This study suggests that MSF stimulates tumour cell migration in an autocrine and paracrine manner, modulated by the type of MSF isoform expressed and by the presence of NGAL and other possible inhibitors. This provides a rational platform for subsequent more extensive investigations of the possible diagnostic and prognostic significance of MSF expression in oral tumours.

Chapter one: Introduction

1.1 Head and Neck Cancer (HNSCC)

Cancer is one of the main causes of morbidity and mortality, with more than 7.6 million deaths yearly worldwide (WHO, 2008; Scully, 2005). Head and neck cancer is the fifth most common cancer worldwide, with an estimated annual global incidence of 500,000-650,000 cases (Parkin *et al.,* 2001; Parkin *et al.,* 2005; Marur and Forastiere, 2008; Jemal *et al.,* 2008). Cancer in the head and neck region includes the nasal cavity, sinuses, lip, mouth, salivary glands, throat, and larynx. The most common form of malignancy is squamous cell carcinoma, which develops in the mucosal linings of the upper respiratory and digestive tracts. This area is further divided into sub-sities of which the oral cavity (including lips) and larynx are the most common sites of cancer. Salivary gland lesions are relatively rare, representing about 3-6% of all tumours of head and neck neoplasms of which approximately 80% are benign (Speight and Barrett, 2002; Mendenhall *et al.,* 2005). The morphological diversity of salivary gland neoplasms complicates the diagnosis and classification of these tumours. Despite significant improvements in head and neck cancer treatment, long-term patient survival has only moderately improved during the last 20 years (Forastiere *et al.,* 2001). Up to 40% of patients with squamous cell carcinoma in the head and neck region present with metastatic disease and survival is dependent on the disease stage (Lefebvre, 2005). My project has primarily focussed on evaluating the diagnostic and prognostic potential of a novel bioactive tumour marker for improving the clinical management of patients with OSCC and salivary gland tumours (SGT).

1.1.1 Oral squamous cell carcinoma (OSCC):

According to the International Classification of Disease (ICD version 10, categories:C00-06) oral cancer refers to a sub-group of head and neck cancers that develop on lips, tongue, gingival, floor of the mouth, buccal surface and other intra-oral locations. Oropharynx cancers arise in tonsil, oropharynx and in some classification systems, in the base of the tongue and posterior soft palate. Salivary gland tumours and tumours of the nasopharynx are excluded from oral and oropharyngeal neoplasms. The term oral cancer is synonymous with malignancies arising from the lining of the oral cavity in any of the above indicated regions.

Squamous cell carcinomas of oral mucosal origin account for more than 80% of all malignant presentations at the aforementioned anatomical sites (Shah, 2003).

Oral cancer is the eighth most frequent cancer in the world among men and the 14th among women, accounting for nearly 3% of all cancer cases worldwide (IARC, 2004). While historically the majority of patients have been found to be over the age of 40 at the time of detection, it may also occur in those under this age. From a gender perspective, although the disease is more frequent in men than in women (1.8:1) the higher sex ratio is slowly narrowing. This is most likely due to lifestyle changes, primarily an increase in smoking habits among women over the latter part of the last century, although firm data on this link are lacking.

There is a wide geographical variation in the incidence of oral cancer (Warnakulasuriya, 2009). It is estimated that more than 750,000 patients worldwide suffer from oral cancer (Parkin *et al.,* 2005). There has been a considerable rise in the number of oral cancers in many countries (Holmstrup *et al.,* 2006), with 275,000 new cases of oral cancer reported in 2002 (Parkin *et al.,* 2005). The areas with highest incidence rates are south and south-east Asia (e.g. Pakistan, Sri Lanka, India and Taiwan), parts of Western (e.g. France) and Eastern Europe (e.g. Hungary, Slovenia), parts of Latin America and the Caribbean (Brazil, Uruguay and Puerto Rico) and Pacific regions (e.g. Papua New Guinea and Melanesia).

In the United Kingdom (UK) oral cancers are less common, accounting for 1.6% of all new cancer cases (Cancer Research Campaign, 2005), with approximately 5400 new cases of oral cancer each year and approximately 1800 deaths (Cancer Research UK, 2007). Rates in Scotland (13.1 per 100.000 populations) are higher than in other parts of the UK, the incidence is around 670 cases per year with a five year survival of 42% for men and 48% for women (Scottish Cancer Intelligence Unit Online 2001; Cancer Research UK, 2007). In Iraq, OSCC account for 91.5% of all oral cancer whilst the incidence reaches 4.5% of all cancer cases according to Iraqi cancer registry (The Iraqi National Cancer Registry INCR, personal communication). Several countries and regions have reported sharp increases in the incidence rates e.g. Scotland, Germany, France, Central and Eastern Europe due to tobacco use and alcohol consumption (Petersen 2005; WHO report, 2004).

The most common sites of oral cancer are the floor of the mouth, the sides and undersurface of the tongue and the back of the throat. The tongue is the most common site for intra-oral cancer among European and the US populations, amounting to 40–

50% of oral cancers (Warnakulasuriya, 2009). It is important to know that the visible part of the tongue, the dorsum, is not a site where SCC develops; rather, it develops on the side (lateral surfaces) and underside (ventral surface), two areas not visible to cursory intra-oral examination. Buccal mucosal cancer is more common among Asian populations due to betel quid/tobacco chewing habits. In Sri Lanka, 40% of oral cavity cancers are found on the buccal mucosa (Rosenquist *et al.,* 2005). Other intraoral sites for mouth cancer include floor of mouth, gingivae and palate.

Survival rates for oral cancer are still very poor with 15% to 50% 5-year survival reported, and these have not improved in the last few decades despite advances in therapeutic interventions and our increased understanding of the disease (Vokes *et al.,* 1993; Parkin *et al.,* 2005). The essential factor in the lack of improvement in prognosis over the years is the fact that a significant proportion of OSCC are not diagnosed or treated until they reach an advanced stage. This diagnostic delay may be caused by either patients (who may not report unusual oral features) or by health care workers (who may not investigate observed lesions thoroughly). It is presumed that such delays are longer for asymptomatic lesions (Speight *et al.* 1992).

Epidemiological and molecular studies of clinical cases suggest a strong association between HNSCC and tobacco use, alcohol consumption, genetic factors and infection by human papillomavirus (HPV) (McKaig *et al.* 1998; Gillison 2004; Stadler and Patel *et al.* 2008; Leemans *et al.* 2011).

1.1.1.1 Etiology of oral cancer

The etiology of OSCC is predominantly related to the following factors:

1.1.1.1.1 Tobacco usage:

The increased risk of developing OSCC is greater in smokers than in non-smokers (Johnson *et al.,* 2000). As it might be expected, the risk of cancer development increases with the number of cigarettes consumed each day, the amount of tobacco previously smoked and the number of years that the habit has been pursued. Ten years after cessation of tobacco use, the risk of developing OSCC declines (Franceschi *et al.,* 1990). Tobacco contains a number of carcinogens (tobacco-specific nitrosamine, polycyclic aromatic hydrocarbons and substances that generate free radical oxygen intermediates) which contribute to cancer initiation and progression as a consequence of the irreversible genetic damage they inflict (La Vecchia *et al.,* 1997).

1.1.1.1.2 Alcohol:

It has been observed that alcohol abuse and OSCC are related. It is considered to be an important risk factor for oral cancer development. Cigarettes and alcohol are, therefore, associated with the development of most OSCC. It is a common observation that heavy alcohol users are also heavy smokers. The synergistic role of alcohol and tobacco increase the risk of malignancy significantly (Maier *et al.,* 1990). All types of alcohol contribute to the cancer risk in proportion to their alcoholic content (Kabat and Wynder 1989). It has been shown that acetaldehyde, the first metabolite of ethanol, is carcinogenic. The role of microbes in the production of acetaldehyde in the oral cavity has previously been described (Homann *et al.,* 2000; Muto *et al.,* 2000; Jokelainen *et al.,* 1996) but one recent study measured the alcohol dehydrogenase (ADH) activity of the viridans group of Streptococci and showed that *Streptococcus salivarius* produced high amounts of acetaldehyde. The observation supports the concept of a novel mechanism of alcohol in the pathogenesis of oral cancer (Kurkivuori *et al.,* 2007). It is believed that possible carcinogenic contaminants and congeners may be present in alcoholic beverages and those drinks may enhance the penetration of carcinogens across the mucosal barrier (Wight and Ogden, 1998).

1.1.1.1.3 Infective agents:

25% of cancer deaths in the developing world and 6% in industrialised countries are caused by infectious agents (Lopez *et al.,* 2006). Candida albicans and some viruses, such as Herpes simplex (HSV), Epstein-Barr virus (EBV) and Human Papilloma virus (HPV) have been implicated in development of oral cancer. Several studies have suggested that transient viral infection exerts an influence on genomic DNA accordingly; the absence of viral particles at the time of presentation does not exclude a viral aetiology (Scully, 1992). The genotypes of HPV most commonly found in oral carcinoma are 16 and 18. The oncogenic potential of high risk HPV is attributable to its ability to insert specific DNA fragments (early genes E5, E6 and E7) into the host cellular genome. As a result of this integration, some key functions of tumour suppressor factors are abrogated (p21, p53 and pRb pathways, respectively), leading to defects in apoptosis, DNA repair mechanisms, cell cycle regulation and, finally, to cellular immortalisation (Szentirmay *et al.,* 2005; Ragin *et al.,* 2007). Robinson *et al,*

(2010) found that the diagnosis of 'HPV related' oropharyngeal SCC is based on high risk HPV DNA detection along with the expression evidence of viral oncogene.

Serological and electron microscope studies of OSCC have demonstrated the presence of these viruses in some cases. While it is debatable that organisms act alone to initiate a cancer, they may act along with other agents like alcohol or cigarette smoke to cause malignant transformation, viruses act as co-carcinogen. Syrj¨anen (2005) found that patients with HPV 16-positive tumours seem to have a better overall- and disease-specific survival, as compared with the HPV-negative group.

Candida albicans is often associated with speckled leukoplakia; such lesions are prone to undergo malignant transformation, because *Candida albicans* produces nitrosamines which are known to be carcinogenic (Krogh *et al.,* 1986).

1.1.1.1.4 Nutrition:

The consumption of fruit and vegetables, particularly those rich in carotene, vitamin C, and vitamin E has been reported to be associated with a reduced risk of oral cancer development, thereby implying that a diet deficient in antioxidants may increase the risk of developing oral and pharyngeal carcinoma and for precancer (Nagao *et al.,* 2000; Petridou *et al.,* 2002; Chainani-Wu, 2002).

1.1.1.2 Genetic changes associated with oral cancer development:

Cancer development is a multi-step process involving (i) *initiation*: the occurrence of a genetic lesion resulting in the inappropriately elevated expression of an "oncogenes" or the inactivation of a "tumour suppressor gene"', and (ii) tumour *progression*: the subsequent accumulation of further such pre-disposing lesions in the clonal progeny of the initiated cell (Nigro *et al.,* 1989; Cowan *et al.,* 1992). Tumour progression accordingly involves the clonal expansion of these increasingly aberrant progeny cells as a result of their acquired relative growth advantage (Nowell, 1976). The requirement for the random accumulation of several predisposing genetic lesions is consistent with the tendency of oral cancers to develop relatively late in life.

Abnormalities in the expression of a wide variety of proto-oncogenes (e.g. *ras*, *myc*, *erbB1* and genes associated with 11q13 amplicon) have been well documented in oral cancer (Prime *et al.,* 1997). With regard to tumour suppressor genes, loss of heterozygosity studies have indicated that early events in cancer development, (these often involving the transitions from normal to dysplasia) are associated with

abnormalities on the short arms of chromosomes 17, 9 and 3 (Califano *et al.,* 1996). A number of tumour suppressor genes have been implicated in the development of oral cancers, including, *p53*, *CDKN2A* and *Rb* gene. For example, p53 inactivation results in loss of a G1 checkpoint. Over-expression of p53 (this taken as an indicator of its mutation and is associated with approximately 40% of oral cancers and 80% of OSCC (Boyle, 1993; Greenblatt, 1994). Taken together, these findings suggest that knock-out of normal p53 function is a common/essential step in oral carcinogenesis. Beside inactivation of tumour suppressor genes, mutation and dysregulated expression of oncogenes may also be involved in the pathogenesis and progression of OSCCs. In this regard, mutations in the *ras* oncogene family result in persistent mitogenic signalling, whereas upregulation of *c-myc* provides an additional proliferative signal resulting in dysregulated hyper-proliferation (Field, 1992; Todd *et al.,* 1997). Amplification and overexpression of the *erb-B* oncogenes have also been found to be correlated with aggressive tumour growth and poor survival in oral cancer (Brand *et al.,* 1995; Werkmeister *et al.,* 1996; Xia *et al.,* 1997).

Wang *et al*., (2006) found that the proteins cyclin D1, Ki-67 and activated extracellular signal-regulated Kinase (ERK1/2) expression, were shown to be significantly higher in OSCCs than in the normal mucosa, both positive expression of the cell proliferation-related index Ki-67 and over-expression of activated ERK1/2 in OSCCs were significantly associated with a moderately or poorly differentiated grade. Miyazaki *et al*., (2006) showed that polypeptide growth factors play important roles in the processes of cell migration and invasion and they showed that transforming growth factor beta (TGF-β) and epidermal growth factor (EGF) differentially affect gene expression in primary and metastatic SCC cells, and likely contribute to the invasive properties of metastatic cells through regulation of both common and specific mediators for each growth factor.

Although it is believed that up to a third of precancerous lesions may eventually evolve into invasive OSCC over a 10 year interval, no reliable histopathological parameters have been indentified that predict for the risk of such malignant transformation. There is therefore a pressing need to identify such a predictive biomarker (Schepman *et al.,* 1999).

1.1.1.3 Clinicopathological aspects:

Early OSCC may present as a white raised or flat patch, red patch or a mixed red and white lesion. The mucosal surface may develop ulceration and with time this ulceration becomes an exophitic mass with a fungating or papillary surface. Other tumours have an endophytic growth pattern that is characterised by a depressed, ulcerated surface with a raised, rolled border (Silverman, 1988), therefore any solitary oral lump, ulcer, white or red lesion persisting for more than three weeks (Brandizzi, 2008) or non-healing socket, numbness, or unexplained loose tooth should be regarded as cancer until proven otherwise (Mashberg and Samit, 1995).

Pain is a common but not a reliable indicator as to whether a particular lesion may be malignant; larger, advanced carcinomas will often be painful, but many early oral cancers will be totally asymptomatic or may be associated with only minor discomfort (Scully and Bagan, 2009).

1.1.1.4 Potential malignant lesions

Oral leukoplakia (white lesion), erythroplakia (red lesion) and erythro-leukoplakia (mixed red and white lesion) are potentially malignant lesions that cannot be clinically or pathologically diagnosed as any other conditions (Axell *et al.,* 1996). In a recent study, 15-20% of non-homogeneous leukoplakia progressed to malignant tumours, whereas only 3% of the homogeneous leukoplakia developed carcinoma (Holmstrup *et al.,* 2006). Although erythroplakia is much less common than leukoplakia, it has been shown to have a higher risk of malignant transformation (Reichart and Philipsen, 2005). Currently, clinicians cannot predict if a mild, moderate or severe dysplasia will progress to oral cancer based on histopathologial morphology alone. However, up to 50% of severely dysplasia lesions progress to carcinoma (Speight and Morgan, 1993) although there is no way of predicting which severe dysplasia will progress. A recent study revealed that there was significant inter-observer variation in the grading of oral epithelial dysplasia (Kujan *et al.,* 2007). Identifying more accurate predictive markers of subsequent neoplastic transformation would greatly assist in the selection of an optimal therapeutic strategy.

1.1.1.5 Biomarkers of Head and Neck cancer:

Advances in the analysis of molecular alterations in cells undergoing malignant transformation have increasingly revealed the mechanisms which have lead to the occurrence and progression of malignancies. The identification of individual molecules that are associated with malignant transformation has led to an ever increasing number of molecular markers that have shown to be related to tumour stage and grading or may be indicative of prognosis and the clinical course of the disease. Many molecular indicators of malignancy have been evaluated in recent years. Characterisation of malignant disease by molecular markers is expected to improve our understanding of the as yet unpredicable clinical outcome for individual patients, as well as identify alternative (non surgical) therapeutic strategies, such as gene therapy (Schliephake, 2003). Although several studies have used different types of biomarkers regarding head and neck squamous cell carcinoma, none have as yet proved to be of sufficient clinical utility. These biomarkers including tyrosine kinase receptors: epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF); matrix metalloproteinases (MMPs); p16, p53, Bcl-2, cyclin D1 (CCND1), and Cox-2, have demonstrated inconsistent and sometimes contradictory results (Brinkman & Wong, 2006; Deshpande and Wong, 2008; Molinolo *et al.,* 2009).

Many studies have been divided the tumour markers into different groups according to their function;(i) tumour growth development: cell cycle acceleration and proliferation, (ii) angiogenesis, (iii) tumour suppression, (iv) tumour invasion and metastatic potential including adhesion molecules and matrix degradation. Table 1.1 shows some of the most important molecular indicators (Schliephake, 2003; Massano *et al.,* 2006; Oliveira and Ribeiro-silva, 2011).

Table 1.1: Potential biomarkers in Head and Neck cancer

Class of marker	Oncogene	Carcinogenesis	Prognosis	References
Tumour growth enhancement (cell cycle acceleration, proliferation)	EGF/EGF-R, C-erb1-4 Or Her-2/neu	Over expression	Poor	Ibrahim1997; Werkmeister,2000; Wilkman,1998; Sakaki,2000;Xie, 1999; 1997;
	Ki-67	Over expression	Less relevant for prognosis	Piattelli,2002; Mishima,1998
	PCNA	Over expression	Less relevant for prognosis	Yue,1997;Schwartz,2000; Sakurai, 2000
Tumour suppression & Anti tumour defence	P53	Over expression	Not valid in predicting prognosis	Piattelli,2002; Horta,2007
	pRb	Lack of expression	poor	Nakahara,2000;Pande,1998; Koontongkaew,2000
Angiogenesis	VEGF/VEGF-R	Over expression	Questionable	Artese,2001;Carlile,2001; Tae,2000
Potential tumour invasion & metastasis	MMP9	Over expression	Poor	Arenas-Huertero,1999; Kurahara,1999; Hong,2000
Intracellular Markers	Cytokeratins 8,18,19	Over expression	Poor	Fillies et al., (2006); Nanda et al, (2011)

1.1.2.6 Diagnosis, Prognosis and Management:

Diagnosis of oral cancer generally relies on the histopathological assessment of haematoxylin and eosin stained paraffin embedded tissue sections (Warnakulasuriya, 2001) According to the degree of squamous cell carcinoma differentiation, three subtypes are defined: (1) well-differentiated squamous cell carcinoma showing more than 75% keratinisation; (2) moderately differentiated squamous cell carcinoma with 25-75% keratinisation; and (3) poorly differentiated squamous cell carcinoma with less than 25% keratinisation (Ferlay *et al.,* 2004). The majority of cases fall into the moderately differentiated group. The relationship between clinical prognosis and histological differentiation is not clear, although a lack of differentiation has been associated with more rapid growth and spread. The OSCC morphological classification by differentiation degree is used to describe the histopathologic specimen (Zarbo and Crissman, 1988). The signs and symptoms of early oral cancers and precancerous lesions are approximately similar and seem to be faint. For that reason, if risk factors such as tobacco use or alcohol abuse are present it is important for the clinician to show extreme caution in dealing with these cases. Studies have shown that areas of tissue which appear clinically altered and were identified at the first assessment as "precancerous" have undergone malignant change during follow-up (Pindborg *et al.,* 1963, 1968; Gupta *et al.,* 1980; Schepman *et al.,* 1998). Some of these alterations, particularly red and white patches, are seen to co-exist at the margins of overt OSCC (Wright and Shear, 1985). From the genetic viewpoint, some of the chromosomal, genomic and molecular alterations found in invasive OSCC are also detected in these disorders as well (Rosin *et al.,* 2000; Van Der Riet, 1994). OSCC can be preceded by the appearance of disorders (such as red or white patches) which have the potential to develop into cancer or enable prediction of the development of cancer elsewhere in the oral cavity. As the cancer develops, the patient may observe the occurrence of a non-healing ulcer. After a while, symptoms may worsen to include bleeding, loosening of teeth, difficulty wearing dentures, dysphagia, dysarthria or development of a neck mass (Neville et *al.,* 2002).

Improvement in the clinical outcome of patients with OSCC depends on the early diagnosis and treatment of the cancerous or pre-cancerous lesion (Dolan *et al.,* 1998; Vernham and Crowther, 1994). Several attempts have found that clinical stage, tumour size, pain from face and neck, age and cervical metastasis are strong

prognostic indicators; It should also be noted that there is a good correlation between TNM classification, as defined by Union for International Cancer Control (UICC) and prognosis (Langdon *et al.,* 1977; Pedersen *et al.,* 1992, Therkildsen *et al.,* 1998).

As the ultimate control of oral cancer depends on prevention and early diagnosis, it is clear that every effort should be made to educate the public about risk factors and early features associated with this disease (Horowitz *et al.,* 1996).

1.1.2.7 Treatment

Treatment of OSCC is inconsistent and depends upon numerous factors including the size and location of the primary tumour, lymph node status, presence or absence of distant metastases, the ability of the patient to accept treatment and their wishes. Surgery and/or radiation therapy remain the principle means for treatment of cancers of the lip and oral cavity (Yamamoto *et al.,* 2006). Treatment of oral cancer is principally surgical. Resection of the tumour may be the treatment of choice if the tumour can be excised with an appropriate margin of normal tissues surrounding the tumour (Edwards and Johnson, 1999). Radiotherapy can be delivered with curative intent (radical radiotherapy), or to improve local control following surgery (adjuvant radiotherapy), or to provide symptomatic relief only (palliative radiotherapy). Ionizing radiation may be delivered as an external radiation beam targeting the tumour (external beam radiotherapy or by direct implanting radioactive source within the tumour (brachytherapy). Radiotherapy is appropriate treatment for some patients particularly those with large tumours with bone involvement (Henk and Langdon, 1985). There is as yet no evidence to suggest the use of chemotherapy alone as a curative treatment for oral cancer. Therefore, administration of chemotherapy in patients with HNSCC is in combination with locoregional therapy (surgery or radiotherapy) (Licitra *et al.,* 2003). Another diagnostic and therapeutic method for oral cancer is photodynamic therapy (PDT) which involves administration of photosensitizing agents to the target lesion, which is then stimulated, usually by short wavelength light. The photosensitizing agents, along with the light, kill cancer cells. These light sensitive chemicals only work after they have been activated or "turned on" by certain kinds of light. Regrowth of the tumour was significantly suppressed by fractionated PDT, with a 24 hour interval appearing to be the optimal fractionation interval (Togashi *et al.,* 2006). The limited depth of penetration of PDT limits its usefulness to field change lesions and superficial cancers.

1.1.2 Normal Salivary glands and salivary gland tumours

1.1.2.1 Normal salivary glands

The salivary glands are exocrine glands that produce and secrete saliva into the oral cavity; saliva is produced at a rate of 600-1500 ml every day and contains different types of organic and inorganic substances. It provides a protective barrier for the teeth and supporting structures of the oral cavity and assists in mastication and swallowing by moistening the oral mucosa as well as dry foods and aids in the digestion of food materials. Salivary gland tissue consists of branching ducts that contain the principal secretory cells, the acinar cells (Ellis and Auclair, 1996; Mayers and Ferris, 2007).The gland is encapsulated by the connective tissues divided into lobules internally, and the gland is composed of three mains types of cells; serous, mucus and myoepithelial cells (Bhaskar, 1990).

1.1.2.1.1 Histological structure of salivary glands:

1.1.2.1.1.1 Cells:

All salivary glands are composed of three mains types of cell (Fig.1.1);

- Serous cells: which are a pyramidal shaped cells resting on a thin basal lamina, have a spherical nucleus located in the basal region of the cell, the most characteristic features of the serous cell are the accumulation of secretory granules in the apical cytoplasm, the cytoplasmic base stains with haematoxylin. While the apical cytoplasm stains with eosin. These cells join together to form a spherical mass of cells known as the acinus with a small central lumen. The serous cells are where synthesis, storage and secretion of proteins occur (Kerr, 1998).

- Mucous cells: which are cuboidal shape cells with a flattened or oval shape nucleus located above the basal plasma membrane, the cells are organised into tubules. Glycoprotiens are the product of these cells, which are used for moistening and lubricating saliva functions. These cells are considered to be poorly stained with haematoxylin and eosin (Bhaskar, 1990).

- Myoepithelial cells (basket cells): these cells are located near the secretory and intercalated ducts portion, the cell body is small with a flattened nucleus and numerous cytoplasmic processes that radiate out to the connective tissue. The function

of the myoepithelial cell is their ability to contract to accelerate secretion of saliva from the lumina of the secretory units and ducts (Kerr, 1998).

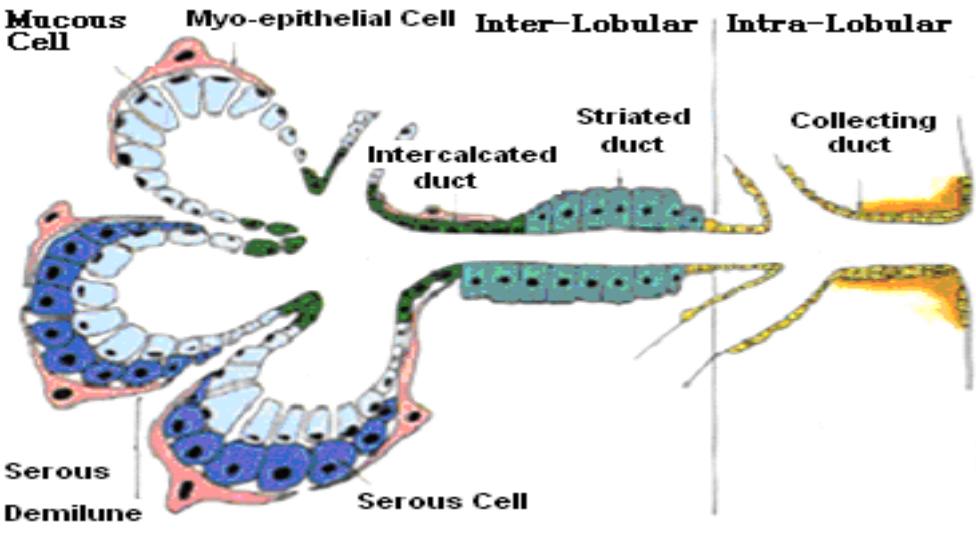


Figure 1.1 Histological structure of salivary gland (Oral Anatomy, Histology and Embrylogy, Berkovilz *et al.*, 2002)

1.1.2.1.1.2 Ducts:

The saliva is drained by a series of ducts, the intralobular segments of the salivary gland duct system consisting of intercalated and striated ducts, which are also known as `secretory' ducts because of their metabolic activity. The intercalated duct lies directly in contact with the acinus and is formed by cuboidal cells with an irregular layer of myoepithelial cells. Several benign and malignant salivary gland tumours are putatively of intercalated duct origin (Chetty, 2000). Normal duct epithelium consisted of two layers: inner columnar cells and basal cuboidal or squamous cells. A few goblet cells are present amongst the inner cells (Takeda and Yamamoto, 1997). An intercalated duct drains into a striated duct, which empties into an excretory duct in the direction of the opening of the salivary gland unit (Hanna and Suen 1998; Mayers and Ferris, 2007) (Fig. 1.1). Basal (reserve) cells line the outer side of excretory ducts. They are relatively isomorphic and have a prominent basal cell layer and distinct basement membrane-like structures (Seifert, 1991).

1.1.2.1.1.3 Connective Tissues:

The connective tissue of the salivary glands surrounds the gland in a capsule and divides the gland into many lobules. It is composed of cells which include; fibroblasts, mast cells, macrophages, fat cells, plasma cells, occasional leucocytes and

collagen fibres which are embedded in ground substance that consists of proteoglycan and glycoprotiens, the blood vessels and nerves supply are also embedded within connective tissues (Douglas, 2000).

1.1.2.1.2 Classification of salivary gland:

Salivary glands have been classified according to their site and location into main groups; a) three pairs of major salivary glands consisting of the parotid glands, submandibular glands and sublingual glands, which are located outside of the oral cavity and have extensive duct systems which deliver saliva to the mouth. b) Minor salivary glands which are scattered in the oral mucosa (Seifert and Sobin, 1992; Mayers and Ferris, 2007).

1.1.2.1.2.1 Major salivary glands:

1.1.2.1.2.1.1 Parotid gland;

The parotid gland is considered the largest salivary gland, located anterior and inferior to the external ear overlying the Ramus of the mandible. It is a bilateral gland found in the subcutaneous tissue of the face. The excretory duct of the parotid gland is called Stensen's duct, which opens within the buccal cavity opposite to the upper second molar teeth, this opening is marked by a small elevated tissue on the inner surface of the cheek called the parotid papilla. The parotid gland receives nerve supply from glossopharyngeal nerve (parasympathetic) through the otic ganglion (Bhaskar, 1990 & Kerr, 1998).

Histologically: The Parotid gland is considered a serous gland (100%). There is extensive connective tissue septa that divides the glands into lobules, the intercalated duct is long and has a small diameter and lumen and there are numerous striated ducts that are lined by columnar cells. Numerous fat cells are also present in the parotid gland which increases in number with age (Bhaskar, 1990; Kerr, 1998). During embryonic development the parotid is seeded with lymphoid cells that develop into several lymph nodes within and around the parenchymal tissue. The lymph nodes are often located in the superficial lobe of the gland. Lymph vessels pass to retromandibular, superficial and deep cervical lymph nodes, which have to be considered when treatment is planned (McKean et al, 1985).

1.1.2.1.2.1.2 Submandibular gland:

A pair of salivary glands is located in the submandibular triangle behind and below the free border of mylohyoid muscle beneath the floor of the mouth. The excretory duct of the submandibular gland called (Wharton's ducts) which opens into the oral cavity at sublingual (caruncles) which is a small papilla on either sides of the lingual frenum on the floor of the mouth near the sublingual gland duct. Secretion by the submandibular gland and other salivary glands is regulated directly by the parasympathetic and indirectly by the sympathetic nervous system. The parasympathetic innervations of the submandibular and sublingual glands input from the facial nerve through the submandibular ganglion, whilst the sympathetic through preganglionic nerves in thoracic segment T1-T3 (Ten Cate, 1998).

Histologically: These glands are enclosed in a well-defined capsule of connective tissue, separating the gland into lobules, which contain the secretory units. They are classified as branching tubuloacinar glands as they contain both serous and mucous secretory cells and the mucous terminal ends are capped by demilunes of serous cells. Although the serous cells are most predominant, the mucous cells are more active, so the main product of the glands is viscous saliva (Ten Cate, 1998; Douglas, 2000). There are no lymph nodes, lymphoid nodules nor large peripheral nerves within the gland. The lymph vessels drain to pre-vascular and pre-glandular sub-mandibular lymph nodes. The lymph vessels run straight through to sub-gastric lymph nodes from the dorsal side of sub-mandibular gland (Mayers and Ferris 2007).

1.1.2.1.2.1.3 Sublingual gland:

A pair of salivary glands is located between the floor of the mouth and the mylohyoid muscle anterior to the submandibular gland, the main excretory duct that opens near the submandibular ducts called Bartholin's duct, 5% of saliva is produced by the sublingual glands (Bhaskar, 1990).

Histologically: Sublingual glands are enclosed by a poorly defined capsule of connective tissue, but the connective tissue septa are clearly present within the gland. It is a mixed gland consisting of mucous cells which are most predominant and serous demilunes and very rare pure serous acini are present. In the sublingual glands, both the intercalated ducts and the striated ducts are relatively short. Lymphoid tissue and large peripheral nerves are not present in the sublingual gland. The lymph vessels lead to submandibular or to deep jugular lymph nodes (Mayers and Ferris 2007).

1.1.2.1.2.2 Minor salivary glands:

These glands are located beneath the epithelium in almost all parts of the oral cavity where there are about 400 to 500 minor salivary glands located in oral mucosa. More than 50% of minor salivary glands are present in the palate and the others are present in the lips, floor of mouth, tongue, and buccal mucosa.

Their ductal system is simpler and the secretory units of these glands secrete saliva which is mostly mucous product directly through short ducts into the mouth, except the Van Ebner's glands (Douglas, 2000).

Histologically: Minor salivary glands are not encapsulated but are separated from one another by connective tissue beneath the mucous membrane. They are considered to be mixed glands consisting of mucous and serous acini, predominantly mucous, contributing about 5% of total saliva secretion (Bhaskar, 1990; Kerr, 1998; Spiro and Spiro 2001).

1.1.2.2 Saliva:

Saliva is a complex fluid present normally in the mouth which is secreted and produced by various salivary glands, it represents about 1/5 of the total plasma volume, and the total volume of saliva secreted daily is between 0.5-1.5 L. The contribution of each type of gland to saliva secretion depends considerably upon the stimulation of various intensities (Herrera et al, 1988; Fox, 1989).

Saliva contains 99% water and the remaining 1% consisting of:

- *Inorganic substances which include;*

Calcium, phosphate, hydrogen carbonate, sodium, potassium, chloride, fluoride, thiocyanate, magnesium, lead.

- *Organic substances include;*

Protein (glycoprotein, mucins), proline rich protein, amylase, lysozyme, lactoferrin, lipase, peroxidase, immunoglobulin (SIgA), histatin, statherin.

- *Serum constituents*; albumin, blood clotting factors, blood groups, amino acids, urea, steroid hormones, ammonia, and sugar.

- *Growth factor [GF]:* Epidermal growth factor [EGF], Nerve growth factor [NGF], Transforming growth factor alpha [TGF-α] and beta [TGF-β], Basic fibroblast growth factor [bFGF], Vascular endothelial growth factor [VEGF], Insulin growth factor [IGF-I] and [IGF-II].

Functions of saliva:

The main functions of saliva shown in Table 1.2:

Table 1.2: The Major functions of saliva

Functions	Salivary components
(1) Protective functions:	
1-Lubrication	Mucins, proline-rich glycoproteins, water
2-Antimicrobial Amylase	Complement, defensins, lysozyme, lactoferrin, lactoperoxidase , mucins, cystatins, histatins, proline-rich glycoproteins, secretory IgA, secretory leukocyte protease inhibitor, statherin, thrombospondin
3-Growth factors	Epidermal growth factor (EGF), transforming growth factor- alpha (TGF-α), transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor (IGF-I & IGF-II), nerve growth factor (NGF)
4-Mucosal integrity	Mucins, electrolytes, water
5-Lavage/cleansing	Water
6-Buffering	Bicarbonate, phosphate ions, proteins
7-Remineralization	Calcium, phosphate, statherin, anionic proline-rich proteins
(2) Food- and speech-related functions:	
1-Food preparation	Water, mucins
2-Digestion	Amylases, lipase, ribonuclease, proteases, water, mucins
3-Taste	Water, gustin
4-Speech	Water, mucins

Adapted from FDI Working Group 10 (1992); Fox (1989); Herrera *et al.,* (1988).

1.1.2.3 Salivary gland tumours

Salivary gland cancers are an uncommon disease of the upper aerodigestive tract. These tumours represent about 3% to 6% of all tumours of head and neck neoplasms. Neoplasms of the salivary gland are a heterogeneous and important group of tumours of Oral and Maxillofacial pathology. Salivary gland tumours may be categorised into benign neoplasms, tumour like lesions, and malignant, they arise from either the major salivary glands (i.e. parotid gland, submandibular gland, and sublingual gland) or in one of the 600-1000 minor salivary glands which are distributed throughout the sub-mucosa of the upper aerodigestive tract. Salivary gland neoplasms present a most diverse group of tumours. They originate from the cells of neuroectoderm or neural crest which have the potential to differentiation into a wide variety of cell types (Shrestha *et al.,* 1994; Mackie *et al.,* 1988). The etiology, prognostic factors and risk factors are poorly defined. Considering variation in histological characteristics, clinical behaviour, pathogenesis and the diversity of anatomic sites, these tumours represent a challenge for head and neck surgeons and pathologists (Cawson, 1978; Ward and Levire, 1998).

Salivary gland neoplasms vary morphologically not only among individual tumours but there is also heterogeneity within the one and the same tumour mass in an individual patient (Eveson, 1992). The difficulty and controversy in the diagnosis and management of salivary gland neoplasms requires a clear understanding of histogenetic concepts for salivary gland tumours, the histomorphology of certain tumours, and their classification in order to overcome this challenge. It is also difficult to differentiate benign from malignant tumours and furthermore the decision as to the best method of treatment depends upon reliable information about survival rates. Prognosis and survival statistics following treatment of salivary malignant tumours have been very difficult to interpret because these are rare tumours with a diverse histology. Besides, the clinical course of salivary gland tumours is generally insidious and a long-term follow-up is required to determine the outcome (Licitra *et al.,* 2003).

1.1.2.3.1 Classification:

Different classification of salivary glands tumours have appeared based on their cytological, architectural, and biological characteristics and recently the

classification has taken into consideration the behaviour and the prognosis of these tumours.

The first well recognised histological classification of salivary gland cancer (SGC) was published in 1953 (Foote and Frazel 1953). The World Health Organisation (WHO) published a classification in 1991 (Seifert, 1991) which has been updated several times, and the most recent version from 2005 (Table 1.3), where 24 salivary gland carcinoma (SGCs) of epithelial origin are included (Barnes *et al.,* 2005). Currently, 24 (SGCs) entities have been described and some are also grouped by degree of malignancy. Carcinoma ex pleomorphic adenoma is classified into three types based on the degree of invasion into the capsule. The low-grade cribriform cystadenocarcinoma was originally described as a low-grade salivary duct carcinoma but has been renamed to avoid confusion with salivary duct carcinoma (Barnes *et al.,* 2005). The secondary tumours of the salivary glands consist usually of metastases from primary squamous cell carcinomas or from melanomas of the skin of the head and neck region.

Benign epithelial tumours:

- **pleomorphic adenoma**
- myoepithelioma
- Basal cell adenoma
- Wartin's tumour
- Oncocytoma
- Canalicular adenoma
- Sebaceous adenoma
- Lymphadenoma, sebaceous and non-sebaceous
- Ductal papillomas
- Cyctadenoma

Soft tissue tumours

- Haemangioma

Lymphoma

- Hodgkin's lymphoma
- Diffuse large B-cell lymphoma
- Extra-nodal marginal B-cell lymphoma (MALT lymphoma)

Table 1.3: WHO histological classifications of malignant salivary gland tumours of epithelial origin, 1991 (Seifert, 1991) and 2005 (Barnes *et al.*, 2005).

Seifert 1991	**Barnes 2005**
Acinic cell carcinoma	Acinic cell carcinoma
Mucoepidermoid carcinoma	**Mucoepidermoid carcinoma**
Adenoid cystic carcinoma	**Adenoid cystic carcinoma**
Polymorphous low-grade adenocarcinoma (terminal duct adenocarcinoma)	Polymorphous low-grade adenocarcinoma
Epithelial-myoepithelial carcinoma	Epithelial-myoepithelial carcinoma
	*Clear cell carcinoma, not otherwise specified
Basal cell adenocarcinoma	**Basal cell adenocarcinoma**
Sebaceous carcinoma	**Sebaceous carcinoma**
	*Sebaceous lymphadenocarcinoma
Papillary cystadaenocarcinoma	*Cystadenocarcinoma
*Low-grade cribriform cystadenocarcinoma	
Mucinous adenocarcinoma	Mucinous adenocarcinoma
Oncocytic carcinoma	Oncocytic carcinoma
Salivary duct carcinoma	Salivary duct carcinoma
Adenocarcinoma	***Adenocarcinoma, not otherwise specified (NOS)**
Malignant myoepithelioma (Myoepithelial carcinoma)	Myoepithelial carcinoma
Carcinoma in pleomorphic adenoma (Malignant mixed tumour)	Carcinoma ex pleomorphic adenoma
	*Carcinosarcoma
	*Metastasising pleomorphic adenoma
Squamous cell carcinoma	Squamous cell carcinoma
Small cell carcinoma	Small cell carcinoma
	*Large cell carcinoma
	*Lymphoepithelial carcinoma
	Sialoblastoma
Undifferentiated carcinoma	
Other carcinoma	

* New entities in 2005, Malignant tumours highlighted in bold have been used in this study

1.1.2.3.2 Epidemiology

Salivary gland tumours are uncommon and represent about 3% to 6% of all tumours of head and neck neoplasms and affect about 1 to 3 per 100,000 people. Salivary gland tumours occur most commonly in the sixth decade of life, benign tumours develop in women more than men above the age of 45, while the malignant tumours have an equal sex distribution after 60 years of age (Ellis & Auclair, 1996). Most of the benign tumours arise in the parotid salivary gland, while 80% of minor salivary glands are found to be malignant (Spiro, 1986; Eveson and Cawson, 1985; Joseph *et al.*, 2003; Barnes *et al.*, 2005) (Table 1.4).

The incidence of malignant salivary gland neoplasms varies significantly in different parts of the world. The Surveillance, Epidemiology and End Results (SEER) and American Cancer Society registries record salivary gland cancers according to the anatomical site of origin, *e.g.*, oral cavity and pharynx, rather than within a single, organ-specific salivary gland entity (Witt, 2005). The latest incidence figure from the USA is 1.2/100 000 (2000-2004) (SEER, 2010). Very high incidence rates have been reported in Greenland (Albeck *et al*., 1992) and among the Canadian Arctic Inuits, 13.5/100 000 person. The high risk of SGC persists after migration to a low incidence area, which indicates that genetic or environmental factors gained early in life are etiologically important (Boysen *et al.*, 2008).

Table 1.4: The percentage of tumours according to the salivary gland types

Salivary gland	benign tumour	malignant tumour
Parotid gland	70- 85%	15 – 34%
Submandibular	8 – 15%	30-55%
Sublingual	1 %	80-90%
Minor salivary	1%	40-85%

1.1.2.3.3 Etiology:

Ionising radiation and certain occupational exposures are associated with an increased incidence of salivary gland cancer. Serum or tissue markers associated with a risk for neoplastic transformation have not been reported.

1.1.2.3.3.1 Radiation

The role of the etiological factors for the development of salivary gland cancer is not well understood, but exposure to ionizing radiation from various sources is clearly associated with these tumours (Takeichi *et al.*, 1983, Little 2001). Several studies have not found any association between the uses of mobile phones and salivary gland tumours. (Johansen *et al.*, 2001; Auvinen *et al.*, 2002; Elwood 2003, Hardell *et al.*, 2004; Kundi *et al.*, 2004).

1.1.2.3.3.2 Occupation, lifestyle and nutrition:

Salivary gland cancer risk increases with certain occupations, for example some types of woodworking, plumbing, asbestos mining, rubber manufacturing, among hairdressers and in personnel employed in beauty shops, but the clinical significance has by no means been established (Swanson and Belle, 1982; Horn-Ross *et al.*, 1997). Muscat and Wynder (1998) found that tobacco use and alcohol consumption are unrelated to salivary gland cancers in a case/control study.

1.1.2.3.3.3 Hormones:

Endogenous hormone receptors, such as estrogen, progesterone and androgen, have been recognised in normal and neoplastic salivary glands, but no clear etiologic nor prognostic pattern has appeared (Jeannon *et al.*, 1999; Dori *et al.*, 2000; Nasser *et al.*, 2003).

1.1.2.3.3.4 Viruses

Several viruses have been associated in the pathogenesis of salivary gland cancer. There is a strong relationship between Epstein-Barr virus (EBV) infection and lymphoepithelioma-like carcinomas; this association appears to be in the region of limited to Eskimo and Asian populations (Iezzoni *et al.*, 1995). EBV has also been detected in undifferentiated salivary gland carcinomas (Hamilton-Dutoit *et al.*, 1991; Gallo *et al.*, 1994). This implies that a specific EBV strain may have a special oncogenic potential in Greenland and Asia than in other parts of the world (Tsai *et al.*, 1996). In contrast, Atula *et al.*, (1998) did not identify EBV, human herpesvirus 8, human papilloma virus (HPV) nor cytomegalovirus presence in salivary gland cancer (Iezzoni *et al.*, 1995).

1.1.2.3.4 Histology and clinical features of salivary gland tumours

1.1.2.3.4.1 Pleomorphic adenoma (PA):

Is the commonest type of the benign salivary gland tumours and mostly affects parotid gland 85% whereas the submandibular and minor salivary glands account for 8% and 7%, respectively. Intraorally, the majority of those tumours are located in the palate. It is considered a mixed tumour and it is called pleomorphic because it is composed of both epithelial and connective tissue components, it arises from intercalated duct cells and myoepthelial cells, (Stennert *et al.*, 2001).

Clinical aspects:

Older female patients, more than males, 40-50 years of age are more likely to be affected. Pleomorphic tumours appear as a single irregular nodular mass with a well defined border, slow growth and are asymptomatic. The tumour is almost mobile except when found in the palate, and because it has a thin and delicate capsule have the ability to grow to large proportions and may project into the surrounding parotid tissue. Pleomorphic adenoma may change into a malignant tumour when associated with long duration of the tumour, recurrent, radiotherapy, increasing age of patient and tumour size, in this case the recurrent lesion appears as a multiple nodules and are less mobile than the original tumour (Joseph *et al.*, 2003).

Histological aspect:

Pleomorphic adenoma in the major salivary glands can be partially capsulated or unencapsulated whereas in minor salivary is unencapsulated. Histologically it has a variable structural appearance, there is a wide variety of epithelial structure ranging from duct like structures, sheet, strands of polygonal shape, spindle or stellate cells with a bland nuclear and small or absent nucleoli in a variable stroma either myxoid, mucoid, cartilaginous or hyaline. Myoepithelial cells are the major components of pleomorphic adenoma which may have two morphologic types either plasmacytoid cells which often tend to aggregate or spindle cells which tend to be arranged in parallel pattern. Squamous metaplasia and keratin forming cells may be also present (Joseph *et al*., 2003; Stennert *et al*., 2001).

1.1.2.3.4.2 Angioleiomyoma (Ang):

Angioleiomyoma is a benign mesenchymal tumour derived from the vascular smooth muscle cells, composed of convoluted thick-walled vessels and smooth muscle fascicles (Weiss *et al.,* 2001). Although it typically involves the superficial

soft tissues of the head and neck (Brooks *et al*., 2002), this tumour is rarely found in the salivary gland. The aetiopathogenesis of Angioleiomyoma in the major salivary gland is unclear. Its predilection for the parotid gland may be attributed to the absence of a well-defined capsule and the presence of thick-walled vascular structures. It is well-known that the parotid gland is in close contact or embraces branches of the external carotid arteries and the internal jugular vein (Ellis & Auclair, 1996).

1.1.2.3.4.3 Mucoepidermoid Carcinoma (MC):

Mucoepidermoid Carcinoma is the most frequent malignant tumour of salivary glands representing approximately 30% of all major and minor salivary gland neoplasms in general (Myers and Ferris, 2007). There is a 3:2 female predilection (Barnes *et al.*, 2005). It arises within the parotid and minor salivary gland in about 2/3 and 1/3 cases respectively and the palate is the common site when the tumour arises within oral cavity. Although it is considered the most common salivary gland malignancy in children, elderly people especially women are also affected in the 5th decade of life, (Brandwein *et al*., 2001). According to the multicellular theory, mucoepidermoid tumours develop in the excretory duct cells while the reserve cell theory proposes that the origin is in the reserve cell of the excretory duct (Hanna and Suen, 1998). Prognosis is influenced by the degree of malignancy, tumour grade, stage and the gender and age of the patient (Pires *et al.,* 2004; Mendenhall *et al.,* 2005).

Clinical Aspect:

Mucoepidermoid carcinoma is divided into low and high-grade malignancy, the clinical features of low grade malignancy are a slowly growing swelling that does not exceed 5cm in size, is fixed, asymptomatic, but with high grade histology tumour the symptoms include local pain, tenderness, trismus, dysphagia, facial paralysis and intraorally the tumours are bluish-red and fluctuant and may metastasizes to the bones, lungs, brain and subcutaneous tissues (Brandwein *et al.,* 2001).

Histological aspect:

Mucoepidermoid carcinoma is characterised by containing well defined cellular elements in different proportions; i.e. Epidermoid cells which are arranged in sheets or nests and mucous secreting cells which are always arranged in a glandular pattern and intermediate cells (Joseph *et al.,* 2003).

Mucin and glycogen free clear cells can also be seen. Cysts are found in different sizes, they are filled with mucus, so rupture of these cysts lead to inflammatory reaction in response to mucus liberation. Histologically the cell types are classified according to the Armed Forces Institute of Pathology (AFIP) criteria proposed by Goode *et al., (*1998*)*; Licitra *et al.,* (2003) and Brandwein *et al.,* (2001) into:

- High grade tumours which are composed of squamous (epidermoid) epithelial and intermediate cells and is poorly differentiated (Total point score >7).
- Low grade tumours which are made up from mucous secreting and epidermoid cells and is well differentiated (Total point score 0-4).
- Intermediate grade in which the tumour histologically falls in-between the other two grades (Total point score 5-6).

Prognosis:

Prognosis of mucoepidermoid carcinoma is dependent upon different factors including the site, grade, clinical stages and the surgery accuracy. The survival rates of low and intermediate grade mucoepidermoid carcinoma for 5 years and 10 years are 70% and 96% respectively, whilst for high grade drops to 45% for 5 years and 25% for 10 years (Brandwein *et al*., 2001) (Table 1.5).

1.1.2.3.4.4 Adenoid cystic carcinoma (ACC):

A malignant epithelial neoplasm of ductal and myoepithelial cells represents 20% of all salivary glands malignancies (Myers and Ferris, 2007). About 75% arise in the minor and 25% in major salivary glands (Spiro *et al.*, 1974; Shah and Pate, 2003). Submandiular gland and minor salivary glands are the most common malignancy sites (Matsuba, *et al.*, 1984). ACC can be also seen in other tissues sites include the lacrimal gland, skin, trachea, breast, and the oesophagus (Fordice *et al.*, 1999). According to the reserve cell theory, adenoid cystic carcinoma originates from the reserve cell of the intercalated duct (Dardick and Burford-Mason, 1993). ACC appears most frequently in the 6^{th} decade of life with no sex predilection. The clinical behaviour of adenoid cystic carcinoma is characterised by slowly growing, recurrences, their clinical course is long with late metastasis (Matsuba, *et al.*, 1984).

Clinical aspect:

The signs and symptoms of the tumour depend mostly on the site of the origin, the classic presentation is a painless, slowly growing mass, mucosal ulceration in minor glands of palate and tongue, but advanced tumour Mendenhall *et al.,* 2005 causing local pain, fixation to the underlying structures, and facial nerve palsy is found in case of parotid gland involvement (Conley & Dingman, 1974; Fordice, *et al.*, 1999).

Histological Aspect:

Adenoid cystic carcinoma has a highly distinctive histological pattern composed of rounded groups of uniform, small, darkly staining cells with uniform round nuclei around many clear spaces containing hyaline and mucoid material which gives the classic Swiss cheese appearance (Szanto *et al.*, 1984).

Perzin *et al.*, (1978) describe the histological pattern of adenoid cystic carcinoma and give three types; the cribriform (cells are arranged in abundant hyalinised eosinophilic stroma as delicate anastomosis cords), the solid (cystic glandular pattern), and the tubular types. Because three histological types can appear in a single specimen and to overcome the problem of polymorphism, a pathologic grading system has been initiated to describe the three pathological grades; (Szanto *et al.*, 1984; Fordice, *et al.*, 1999)

Grade one: tubular and cribriform only without any solid component. (Best survival).

Grade two: mostly cribriform and less than 30% solid component.

Grade three: solid type predominantly (poor prognosis).

Prognosis:

The survival rate of the adenoid cystic carcinoma is 89% at 5 years, 67% at 10 years, and 40% at 15 years (Fordice *et al.*, 1999). The 5-year survival of patients with distant metastases is 20 % (Bradley, 2004). The local recurrence rate ranges from 16% to 85% in several series. Chen *et al.*, (2008) studied 46 patients with ACC of whom 12 (26%) had a late recurrence after a 5-year follow-up. The 5-, 10- and 15-year survival rates are disappointing, about 75%, 40% and 8–25% respectively (Spiro *et al.*, 1974, Szanto *et al.*,. 1984, Hamper *et al.*, 1990, Kokemueller *et al.*, 2004). The tumour shows frequent recurrences and the distance metastasis is late. Negative factors influence prognosis including tumour size greater than 4 cm, presence of more than 30% of solid type within the tumour which may associate with poor prognosis (Table 1.5).

1.1.2.3.4.5 Adenocarcinoma (AC):

Adenocarcinoma represents "any malignancy arising from salivary duct epithelium or within salivary glands of epithelial origin" (Regezi and Sciubba, 1999). It accounts for 5-10% of all salivary gland tumours and 16.8% of all salivary gland malignancies, 90% arise in the parotid gland and the remainder (10%) are in the palate, submandibular gland, buccal mucosa, and minor salivary glands. The term Adenocarcinoma is less commonly used as a specific diagnostic entity, because most classification systems have broken down this complex group of neoplasms into separate entities. After recognition of polymorphous low grade adenocarcinoma, salivary duct carcinoma, epimyoepithelial carcinoma the small remaining group of salivary carcinomas with no specific designation may be categorized as Adenocarcinomas (Matsuba *et al.*, 1988; Regezi and Sciubba, 1999).

Clinical Aspect:

The clinical characteristics of adenocarcinoma are that they enlarge slowly, are solitary, painless masses (2-8 cm) which are fixed to the underlying structures, when they arise in the palate there are visible ulcerative and hemorrhagic areas (Spiro *et al.*, 1989; Matsuba *et al.*, 1988).

Histological Aspect:

Adenocarcinoma is an aggressive, high grade malignancy. The neoplastic cells exhibit different morphology including cuboidal, columnar, epithelioid, polygonal, and plasmacytoid, there may also be clear or oncocytic cells. Architectural diversity is represented by various degrees of differentiation consisting of sheet, nests, islands glandular or ductal pattern (Ellis & Auclair, 1996).

Prognosis:

Several factors indicate the prognosis of adenocarcinoma including histological types, perinural and vascular invasion, recurrence, and metastasis. The tumours of the oral cavity have more favourable prognosis than tumours of the parotid and submandibular glands and solid histologic type and perineural invasion seem to be independent clinicopathological factors involved with shorter survival. Patients with low grade tumours have a better prognosis than high grade, due to less cervical lymph node involvement and distance metastases (Ellis & Auclair, 1996) (Table 1.5).

Table 1.5: Malignant salivary gland incidence and survival age according the tha age and origin

Type of tumour	Incidence	Average age	Origin	Survival rate (prognosis)
Mucoepidermiod	35%	47	Excretory duct	Low& intermediate: [70-90% (5-10 ys)] high Grade: [45-25% (5-10 ys)]
Adeniodcyctic Carcinoma	40%	50	Intercalate duct	89% 5 years 67% 10 years 40% 15 years
Adenocarcinoma	10%	58	Intercalate duct	good prognosis for oral cavity tumours and poor for others.
Basal cell adenocarcinoma	1%	60	basaloid epithelial cells	good prognosis
Sebaceous carcinoma	>1%	55	ectopic sebaceous cells	62% 5 years

1.1.2.3.4.6 Basal cell adenocarcinoma (BcAC):

Basal cell adenocarcinoma is avery rare tumour of the major salivary gland and is believed to be the malignant counterpart of basal cell adenoma. It appears microscopically similar to the basal adenoma, except that it exhibits an infiltration growth pattern and has the ability to metastasise. These tumours are composed of nest, cord and solid zones of basaloid cells. Two cytological types of cells are frequently seen, small compact cells and larger polygonal cells. The former may frequently be seen surrounding the latter, often in a palisades fashion. The feature that distinguishes this tumour from the basal cell adenoma is the observation of small nests of neoplasm in adjacent normal structures. Infiltration of the nerve is also seen. Local recurrence and distant metastasis seem to be a distinct potential for basal cell adenoma. This tumour is generally regarded as a low-grade malignancy. With adequate surgical treatment, patients should have a favourable outcome. Most tumours are

asymptomatic but may be associated with pain, tenderness and swelling (Joseph *et al.*, 2003).

Macroscopy:

Basal cell adenocarcinomas most frequently occur in the superficial (lateral) lobe of the parotid gland. The cut surface has a variable colouration of grey, tan-white, or brownish. The texture is homogeneous although some tumours are focally cystic. They are unencapsulated, but some tumours appear well-circumscribed, while others are obviously infiltrative.

Histopathology

Basaloid epithelial cells, which vary from small, dark cells to larger, paler stained cells, form histomorphologic patterns that are described as solid and membranous.

1.1.2.3.4.7 Sebaceous carcinoma (SC)

Sebaceous carcinoma is a rare malignant tumour mainly involved in the ocular adnexae, skin and salivary glands. Sebaceous tumours of the salivary glands are classified into five categories: sebaceous adenoma, sebaceous lymphadenoma, sebaceous adenocarcinoma, sebaceous lymphadenocarcinoma and sebaceous differentiation in other tumours (Ahn and Park, 2006). Approximately 37 cases of salivary gland sebaceous carcinoma had been described; most cases involving the parotid gland and a few appear in the submandibular gland (Ahn & Park 2006; Altemani *et al.*, 2008). Because of its rarity, clinicopathological characteristics and the histogenesis are not fully understood particularly at skin sites (Gnepp, 2005). The parotid is the most common extraorbital site accounting for 30% of all cases (Gnepp, 2005). The neoplastic tissue probably originates from ectopic sebaceous cells, and in fact these normal cells are more frequently found in the parotid than in the submandibular and sublingual glands. The tumour may arise from ectopic sebaceous cells that are displaced in the parotid gland during embryologic development. Like Fordyce granules that develop mainly after puberty, sebaceous cells in the major salivary glands are more common in adults. Takata *et al*., (1989) also suggested that the presence of sebaceous, ductal and mucous cells indicates the origin of SC from pluripotential duct cells. Therefore it is reasonable to suggest that SC, particularly in

children, arises from undifferentiated cells from ductal structures, rather than from ectopic sebaceous cells.

Clinically: the symptoms vary from any indolent, slow-growing, painless and asymptomatic mass, to a painful rapidly progressing swelling accompanied by facial paralysis (MacFarlane *et al*., 1975). If the sebaceous tumour is associated with lymphatic structures, it will be named lymphadenoma or lymphadenocarcinoma.

1.1.2.3.5 Immunohistochemistry for Salivary tumour diagnosis

Salivary gland tumours have complex cytopathological features, so the cytologic criteria of the salivary gland neoplasms are not obvious. The diversity of salivary gland neoplasm morphology from one tumour to other and even within the same tumour makes the recognition of many of these tumours impossible by microscopy alone. Immunohistochemistry (IHC) is a globally available tool that complements histopathological analysis by detecting gene expression at the protein level (Oliveiro and Ribeiro-Silva, 2011)**.** The technique of immunohistochemistry has supplanted a specific diagnostic problem through its general role in salivary gland tumours especially in problematic cases and because the histological appearance does not always correlate with biological behaviour, immunohistochemical staining might give a new measure for understanding and predicting the behaviour of a tumour. Immunohistochemistry uses antibodies to determine the expression of cellular proteins in tissue specimens. The site of this expression is associated with cell structures or cell functions. For analysis of cell differentiation, a variety of new immunohistochemical markers for different cell types have been introduced during the recent years. An appropriate combination of them aids diagnostics and differential diagnostics of diverse histological entities (Agra *et al.,* 2008). There are three main types of epithelial cells of the salivary glands which can be distinguished by tumour markers: acinic cells, ductal cells and myoepithelial cells (deAraujo *et al.,* 2000). Cells can be identified by characteristic markers found on their surface in order to distinguish between benign and malignant tumours and within the different types of salivary gland tumours. Holst *et al.* (1999) and Gnepp and el-Mofty, (1997) suggested that the expression of the product of the C-kit proto-oncongene (CD117) (transmembrane tyrosine kinase receptor) and glial fibrillary acidic protein (GFAP) may help in distinguishing some salivary gland tumours which are share

morphological features, for example, glial fibrillary acidic protein (GFAP) is detected immunohistochemically in a considerable percentage of pleomorphic adenoma (Curran *et al.,* 2001) whilst in polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma expression is low, so this positive result suggests it might be a quite helpful marker. Detection of protein expressed by the c-kit proto-oncogene (CD117) which is negative in normal salivary gland cells (Jeng *et al.,* 2000) and positive in the glandular cells of various types of salivary gland tumours, can be considered as a useful marker for diagnosis of adenoid cystic carcinoma rather than for polymorphous low-grade adenocarcinoma (Holst *et al.,* 1999; Jeng *et al.,* 2000). Functional markers have been associated with survival; e.g. expression of Ki-67 is a well-known prognostic marker in salivary gland cancer (Ben-Izhak *et al.,* 2008).

Recently immunohistochemistry can be used to differentiate between luminal (acinar and ductal cells) and abluminal cells (myoepithelial and basal cells) which can help in understanding the complexity of salivary gland tumours and aids in diagnosis (Cheuk and Chun, 2007).

1.1.2.3.6 Biomarkers used in the diagnosis and prognosis of salivary tumours:

1.1.2.3.6.1 Biomarkers in Saliva

The use of saliva as a diagnostic medium has increased in the last 10 years in US, in preference to peripheral blood, due to several advantages e.g. ease of collection, storage and most molecules that are present in the blood and urine are also identified in salivary secretions. Many studies demonstrated that these salivary markers are reliable and might also be used in post-operative follow up of the patients (Zelles *et al.,* 1995).

The diagnostic and prognostic factors of the salivary gland tumours are dependent on many variables. Numerous studies have concentrated mainly on cytokeratin, actin, S100, vimentin and carcino-embryonic antigen, but none of these markers has proved reliably specific to be used in a diagnostic context. Other attempts have been made by several authors to identify significant diagnostic factors, these studies have taken into consideration clinicopathological features, location of tumours, type and duration of the symptoms, perineural and vascular invasions, histological subtype and age of the patient. Several studies have shown that clinical, histological, and some immunohistochemical markers for prognosis of salivary gland

tumours such as p53, p63, Maspion, (Edwards *et al.,* 2004; Baily *et al.,* 2006), cell proliferation (ki-67, PCNA), NM 23 protein, Mucin (MUC1, MUC4, MUC5AC, MUC5B), CA 125, c-erbB-2, bcl-2 have been associated with patient survival and tumour metastasis (Alos *et al.,* 2005; Do Nascimento *et al.,* 2006).

Speight and Barrett, (2002) found that clinical stage, particularly tumour size, is a critical factor in determining the outcome of salivary gland tumours, and is possibly more important than histological grade for prognosis because large malignancies tend to have a poor prognosis despite of grade and even high-grade tumours may do well when they are small. The 4 cm rule has proved to be a useful clinical guide to behaviour and outcome (Renehan *et al.,* 1999; McGurk, 2001).

The salivary gland and their secretion of saliva is considered to be the natural reservoir of growth factors (GF) and many other regulatory peptides like epidermal growth factor (EGF), several studies recently showed that transforming growth factor alpha (TGF-α) and beta (TGF-β), fibroblast growth factor(FGF), insulin, insulin like growth factor(IGF-I, IGF- II) and vascular endothelial growth factor (VEGF) were identified in the salivary gland which synthesised and secreted them into saliva, so the level of these factors in saliva will reflect the amount present in the salivary gland. Vallejo *et al.,* (1984) and Fekete *et al.,* (1993) have suggested that the salivary gland is a source of growth factors in saliva derived from serum through movement of serum macromolecules via the salivary glands. Purushotham *et al.,* (1995) found that synthesis of growth factors is primarily performed by ductal cells of salivary glands.

The existence of these factors in the salivary gland raises many questions about the effect of these factors on the salivary gland cells and how these factors could be used as diagnostic or prognostic markers.

Table 1.6: Potential biomarkers in salivary gland tumours

Class of marker	oncogene	SGT	Significance/Association	Refernces
Growth factor and their receptor	EGF(R)	SDC, ACC & MC	over expression associated with poor prognosis	Press *et al.,* 1994; Etges et al., 2003;Dodd and Slevin, 2006
	VEGF(R)	ACC, histological grading of mucoepidermoid carcinoma	Neck node metastasis, worse survival, and poor local control of the disease.	Lim *et al.,* 2003; Lequerica-Fernández *et al.,* (2007)
Cell proliferation markers	Ki-67	MEC, ACC, CXPA, acinic cell carcinoma	Prognostic marker, poor survival. Questionable	Luukkaa *et al.,* (2006); Dodd and Slevin, 2006
	PCNA	ACC, CXPA, MEC, PA	prognostic & diagnostic value,	Trendell *et al*., (1997); Young, (1994)
Cell cycle tumour suppressor genes	P53	SGC, MEC	Limited value asprognostic marker mutations	Lim *et al.,* 2003; Alves *et al.,* 2004
	Maspin	ACC, MEC, CXPA	prognostic value	Schwarz *et al.*, (2007); De Lima Navarro, (2004)

MC, mucoepidermoid carcinoma; ACC, adenoid cystic carcinoma; SDC, salivary duct carcinoma; CXPA, carcinoma ex pleomorphic adenoma; SCC, squamous cell carcinoma; PA Pleomorphic adenoma.

1.1.2.3.6.2 Biomarkers in tissue/tumours (Histological section)

In order to facilitate appropriate prognosis and estimating a preferable treatment modality many attempts have been made by several authors in order to perform comparative analysis of biological, pathological, and clinical characteristics of salivary gland tumours. Multiples prognostic biomarkers have been studied by many authors including kallikrein 6, ki-67, proliferating cell nuclear antigen (PCNA), c-erbB2, P53, osteopontin, bcl-2, E-cadherin and DNA content in order to determine whether there was a correlation between the expression of these proteins and patients outcome. Furthermore, the oral mucosa is considered to be the site of mechanical and chemical trauma from mastication, ingestion of hard food and pathological processes, so the maintenance of mucosal surfaces from the external environment exposure and repair process of the oral soft tissue wound requires the presence of growth factors for proper rates of healing. Tumour markers that can be recognised in saliva may be potentially useful for screening for malignant diseases. Salivary diagnosis may be part of a comprehensive diagnostic sheet that will supply improved sensitivity and specificity in the finding of malignant diseases and will aid in monitoring the effectiveness of treatment. Additional studies are certainly required to find out which salivary markers can be used for these diagnostic purposes, and to determine their diagnostic value in comparison with other, more established, diagnostic tests. Table 1.6 provides basic information regarding a number of cytokines relevant to salivary gland tumours.

1.1.2.3.7 Diagnostic aspects

A salivary gland neoplasm must be considered in any patient who presents with a painless, slowly growing swelling at the site of the parotid, submandibular or sublingual glands. Especially at the early stage, malignant and benign tumours are clinically indistinguishable. The overall detection rate for a malignant parotid gland tumour based on clinical findings is approximately 30% (Wong, 2001). The mass may have been present for years in the case of a benign tumour or a low-grade salivary gland cancer. It is remarkable that adenoid cystic carcinoma usually present as a slow-growing mass. Inflammatory disease may be associated with pain and intermittent swelling. Obstruction in the parotid gland is much less frequent than in the submandibular gland; thus, a painful mass in the parotid gland may be a sign of

malignant disease (Spiro and Spiro, 2001). Although both benign and malignant tumours may present with pain, this is not common (Witt, 2004). Findings indicating malignancy are rapid enlargement, palpable cervical lymph nodes, paresis of all or part of the facial nerve, presence of associated lymphadenopathy and fixation of the tumour to the overlying skin or deep structures (Spiro and Spiro, 2001; Wong, 2001 and Shah and Patel, 2003). Salivary gland cancer often spreads by infiltrating into the surrounding structures as nerves, bone, muscles and skin. Parotid gland cancer may infiltrate the facial nerve and invade perineurally to the base of the skull. Regional lymph node involvement is an important route for metastasis in most histological types of tumours.

1.1.2.3.8 Clinical examination

The problem regarding salivary gland malignancies is the difficulty in assessing the malignancy clinically. Locoregional staging of malignant tumours is based on clinical examination in combination with imaging. For lesions in the superficial parotid and the submandibular gland, Fine needle aspiration cytology (FNAC) and sonography are a suitable combination for initial assessment (Lee *et al.*, 2008). Contrast enhanced computer tomography (CT) or/and magnetic resonance imaging (MRI) of the primary site and the neck and carried out in selected cases (Okahara *et al.*, 2003). The objective of imaging studies is to identify accurately the location and extent of the tumour and to recognize whether the lesion is intraglandular or has extraglandular extension (Shah and Patel, 2003). Computed tomography of the chest should be performed to exclude the possibility of distant metastases, if needed (Bradley, 2001). The possibility of distant metastases is associated with tumour grade and is most common in patients with adenoid cystic carcinoma, high-grade mucoepidermoid carcinoma, salivary duct carcinoma and tumours of the submandibular gland, posterior part of the tongue and the pharynx (Bradley, 2001). In general, FNAC is taken preoperatively of all salivary gland tumours (Atula *et al.*, 1995; 1996; Al-Khafaji *et al.*, 1998; Filopoulos *et al.*, 1998 and Chhieng *et al.*, 2000). Surgical biopsy should not be performed on any of the major salivary gland neoplasms, in order to avoid the risk of facial paresis and tumour spread. Further imaging studies are performed postoperatively depending on the tumour type. If a diagnosis of a malignant tumour is made from the surgical specimen in addition to the histological diagnosis, the TNM classification is the only significant prognostic factor

of salivary gland cancer. The TNM classification system is based on tumour size (local extension of the tumour), metastasis to regional lymph nodes and distant metastases. The most recent changes to the TNM classification relate to a revision of the definition of T3 (Sobin and Wittekind, 2002) and the division of T4-tumours which have been divided into T4a (moderately advanced local disease) and T4b (very advanced local disease), leading to the stratification of Stage IV into Stage IVA (moderately advanced local/regional disease), Stage IVB (very advanced local/regional disease) and Stage IVC (distant metastatic disease) (Edge, 2010). Tumours arising in the minor salivary glands are classified according to the criteria for other carcinomas by anatomic site of origin (Sobin and Wittekind, 2002).

1.1.2.3.9 Treatment:

The treatment of salivary glands tumours depends upon numerous factors including whether it is benign or malignant, site of origin, the size, the location, and the extent of the tumours, in order to avoid multiple risk factors that may occurs from incorrect choice of the treatment, for example; extension of the tumour via defective capsule and inadequate initial removal may causes recurrence of the tumour, nerves damage may happened in perineural invasion or during surgical resection of tumours. The main purpose of the treatment of salivary gland carcinoma (SGC) is disease control and preservation of nerve, vascular and muscle function and secretion of saliva. Despite thorough preoperative assessments, very often the diagnosis of malignancy is not known prior to surgery.

Generally the treatment of choice for salivary glands tumours are either medicine likes steroids (Haemangioma) or surgical removal of most benign tumours (Ward & Levier, 1998). Surgery is the main treatment for SGC. Due to anatomical limits of each site, the feasible margins vary to a large extent. Eisele and Kleinberg, (2004) recommend adjuvant radiation therapy for patients at high risk; Radiation therapy affects the function of the salivary glands. Buus *et al.*, (2006) reported that half of the parotid gland function was lost when the radiation dose is 30 Gy. At doses below 25 or 30 Gy, recovery is substantial and the function returns to pre-treatment levels within two years after radiotherapy (Li *et al.*, 2007).

1.2 Migration stimulating Factor

Schor *et al,* (1980-1988) first identified Migration-stimulating factor (MSF); in studies comparing the behavior of different cell types in the collagen gel migration assay (Schor, 1980; Schor *et al.,* 1985a; 1988a). They reported that (i) foetal fibroblasts migrated into a 3-dimensional collagen matrix to a significantly greater extent than did their normal adult counterparts, (ii) this behavioural difference resulted from the production of a soluble "migration stimulating factor" (MSF) by the foetal, but not adult cells, and (iii) tumour-derived and skin fibroblasts obtained from patients with various types of cancer resembled foetal cells with respect to their elevated migration and production of MSF (Durning *et al.,* 1984; Schor *et al.,* 1985b, 1988b; Haggie *et al.,* 1987). Normal adult fibroblasts did not produce MSF or an inhibitor of MSF; the migration of these cells was however stimulated by conditioned media from foetal and cancer patient fibroblasts, thereby providing a sensitive bioassay for the subsequent purification of MSF. MSF was eventually cloned and, most unexpectedly, shown to be a genetically truncated isoform of fibronectin (Schor and Schor, 2001; Schor *et al.,* 2003).

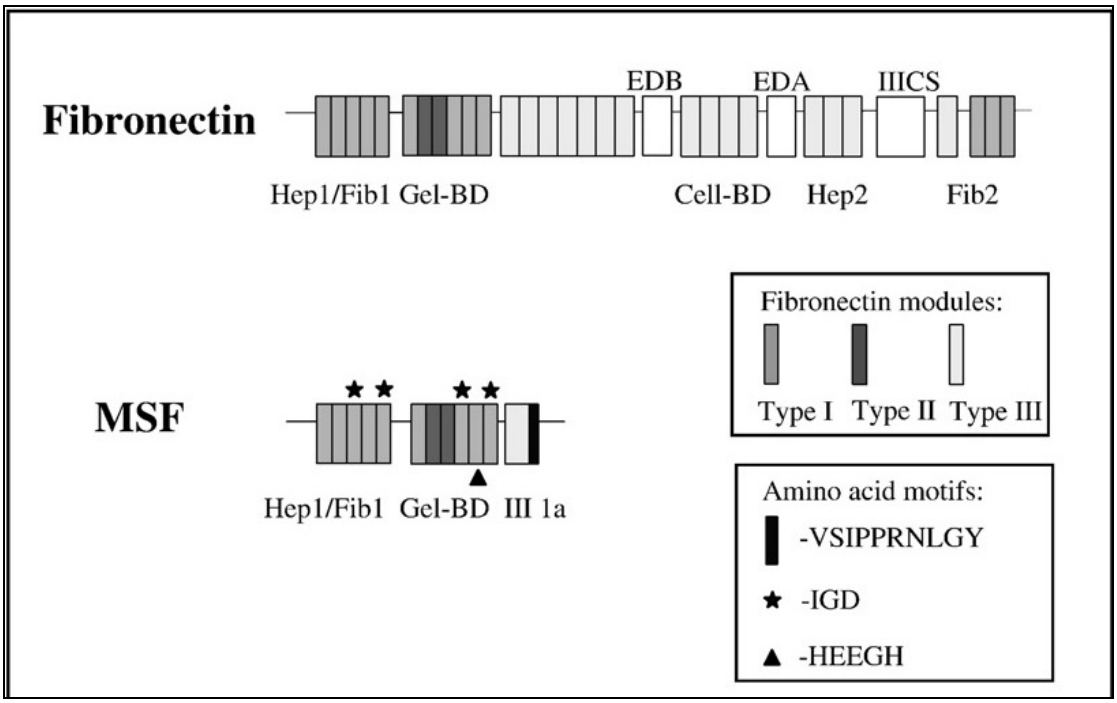
Fibronectin is a ubiquitously distributed macromolecule, present as an insoluble constituent of the extracellular matrix (ECM) and as a soluble component of serum (Hynes, 1990). Both forms consist of two similar, but not necessarily identical, polypeptide chains (with individual molecular masses in the region of 260 kDa) covalently linked by disulphide bonds at their respective C-termini. Each protein chain consists of a tandem array of "functional domains" defined on the basis of their binding proclivities for other matrix molecule and integrin receptors on the cell surface (Fig1.2). Starting at the N-terminus, these include: the Fib1/Hep 1- binding domain (exhibiting affinity for fibrin and heparin), the Gel –binding domain (affinity for collagen/gelatin), the cell binding domain (affinity for certain cell surface integrins such as α5b1), the Hep 2- binding (affinity for Heparin) and the C-terminal Fib2 – binding domain (affinity for fibrin). Each of these functional domains consists of different combinations of three "structural homology modules" designated types I, II and III. This modular structure is reflected by a corresponding modularity in the fibronectin gene, in which types I and II modules are each coded by a single exon, whilst the majority of type III modules are coded by two exons (designated "a" and

"b"). There are approximately 20 previously described full length fibronectin isoforms, all having molecular masses in the region of 260 kDa. These are generated by alternative splicing of the primary fibronectin gene transcript involving the retention or deletion of two particular type III exons (EDA and EDB), as well as a more complex splicing repertoire within the down-stream IIICS region (Hynes, 1990) (Fig 1.2).

Fibronectin is a multifunctional molecule interacting with the cell surface, as well as various other macromolecular constituents of the extracellular matrix. A number of studies involving both limited proteolytic digestion and electron microscopy have revealed that each polypeptide chain consists of a linear array of discrete protease-resistant globular domains joined together by short stretches of extended protease-sensitive linking sequences. Functional analysis of these protease resistant domains indicates that they each express characteristic and discrete affinities. For example, digestion of fibronectin with thermolysin yields at least five peptides referred to as Hep-1/Fib-1 (29kDa), gel (43kDa), Cell (110kDa), Hep-2 (30kDa) and Fib-2(20kDa) in accordance with their respective affinities for heparin (Hep), fibrin(Fib), gelatin (Gel) and the cell surface (cell) (Zardi *et al.,* 1985)

In vitro, fibronectin is synthesised by a variety of cultured cells where it is often incorporated into the extracellular matrix. *In vivo*, fibronectin has a ubiquitous distribution, it is a prominent component of plasma, other fluids and connective tissue matrices.

Fibronectin receptors have been shown to include a multiplicity of integrin dimers. Integrin family members currently known to interact with fibronectin include: $\alpha 3\beta 1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha v\beta 6$ and $\alpha_4\beta_7$ (Yamada *et al.,* 1996).



MSF

MLRGPGPGLLLLAVQCLGTAVPSTGASKSKRQAQQMVQPQSPVAVSQSKPGCYDNGKHYQINQQWERTY
GNALVCTCYGGSRGFNCESKPEAEETCFDKYTGNTYRVGDTYERPKDSMIWDCTCIGAGRGRISCTIAN
CHEGGQSYK**IGD**TWRRPHETGGYMLECVCLGNGKGEWTCKPIAEKCFDHAAGTSYVVGETWEKPYQGWM
VDCTCLGEGSGRITCTSRNRCNDQDTRTSYR**IGD**TWRKKDNRGNLLQCICTGNGRGEWKCERHTSVQTT
SGSGPFTDVRAAVYQPQPHPQPPPYGHCVTDSGVVYSVGMQWLKTQGNKQMLCTCLGNGVSCQETAVTQ
YGGNSNGEPCVLPFTYNGRTFYSCTTEGRQDGHLWCSTTSNYEQDQKYSFCTDHTVLVQTRGGNSNGAL
HFPFLYNNHNYTDCTSEGRRDNMKWCGTTQNYDADQKFGFCPMAAHEEICTTNEGVMYR**IGD**QWDKQHL
GHMMRCTCVGNGRGEWTCIAYSQLRDQCIVDDITYNVNDTFHKR**HEEGH**MLNCTCFGQGRGRWKCDPVL
CQDSETGTFYQ**IGD**SWEKYVHGVRYQCYCYGRGIGEWHCQPLQTYPSSSGPVEVFITETPSQPNSHPIQ
NAPQPSHISKYILRWRPVSIPPRNLGY

Figure 1.2: The structure of fibronectin and MSF and the sequence of MSF and its component parts. Functional domains of fibronectin; Hep1/Fib1, Gel-BD, Cell-BD, Hep2 and Fib2 with each domain consisting; 3 structural modules type I, II and III. The structure of MSF is also shown indicating similarity of Hep1/Fib1 and Gel-BD with a unique 10 amino acid sequence (Schor and Schor, 2001; Ellis *et al.,* 2010)

1.2.1 Modular Structure of MSF and FnI-type:

Migration-stimulating factor (MSF) is a soluble genetically truncated isoform of fibronectin and a potent oncofoetal regulatory molecule. MSF message is transcribed from the fibronectin gene by an unusual "failure" of normal alternative splicing involving read-through of the intron 12 separating exons III-1a and III-1b, and subsequent premature transcript cleavage (Schor *et al.,* 2003). The retained intronic sequence contains a 30 bp in-frame coding sequence (immediately contiguous with exon III-1a), followed by several in-frame stop codons and a cleavage/poly-

adenylation signal. The resultant MSF protein is consequently a truncated isoform of fibronectin, identical to its 70 kDa N-terminus, up to and including the amino acid sequence coded by exon III-1a, and ends in a unique, intron-derived, 10 amino acid sequence not present in any previously identified "full-length" fibronectin isoform (Fig 1.2). As is the case with many cytokines and stress response molecules, MSF message exhibits an extremely short half-life as a consequence of an AU rich instability element in its 3'-UTR (Schor *et al.,* 2003; Bakheet *et al.,* 2001; Chen et al 1994).

The MSF originally cloned by Schor *et al.,* 2003 was essentially identical to the N- terminus of fibronectin consisting of 9 type I modules, two type II modules including heparin/fibrin (Hep1/Fib1) and gelatin (Gel-BD) binding domains of fibronectin and half of the first fibronectin type III module, with the exception of an MSF-unique C-terminal 10 amino acid sequence (Fig 1.2).

The MSF N-terminal fragment is made up of 1–5FnI; the C- terminal region contains 6FnI, 1FnII, 2FnII, 7FnI, 8FnI, 9FnI and part of 1FnIII. The structure of each Fn I module consists of two beta sheets formed by five β-strands and with a hydrophobic core. Two disulfide bonds help in maintaining the structure of Fn I and the anti-parallel β-sheets are stabilised by three amino acids forming a short loop. Four out of nine of the MSF FnI modules contain IGD (Isoleucine-Glycine-Aspartate) amino acid motifs referred to as 3FnI, 5FnI, 7FnI and 9FnI. *In vitro* mutagenesis data indicate that motogenic effect of MSF on fibroblasts is mediated by two IGD amino acid motifs located in MSF modules 7FnI and 9FnI. Significantly, neither of these potent MSF bioactivities is expressed by full-length fibronectin; the mechanism responsible for this crypticity is not known, but is likely to result from steric hindrance blocking the accessibility of the two IGD motifs. On the other hand these studies have been shown also that mutations of IGD to DGI in 7FnI and 9FnI eliminates the motogenic activity of MSF on fibroblasts (Schor *et al.,* 2003).

Two isoforms of MSF have been cloned. These differ by a 15 amino acid deletion (FYSCTTEGRQDGHLW) in the first type II fibronectin structural module and are referred to as MSF+aa (AJ535086-NCBI/PubMed) and MSF-aa (AJ276395-NCBI/PubMed). The term MSF will be used to denote both isoforms. Both isoforms contain the same MSF-specific 10 amino sequence and IGD functional domains. Both isoforms display the same spectrum of potent bioactivities and these are effectively inhibited by PEPQ function-neutralising antibodies. MSF+aa and MSF-aa differ in

terms of their inhibition by NGAL (neutrophil gelatinase-associated lipocalin) which is present in serum and certain tumours (Jones *et al.,* 2007): MSF+aa is inhibited and MSF-aa is not.

1.2.2 MSF Bioactivity.

Recombinant MSF exhibits a broad spectrum of bioactivities, including the (i) stimulation of fibroblast, epithelial and endothelial cell migration, (ii) Up-regulation of Hyaluronic acid (HA) synthesis by target fibroblasts, (iii) Angiogenesis, (iv) Proteolysis (Schor and Schor; 2001; Schor *et al.,* 1989, 2003; Houard *et al,.* 2005). These activities are unusually potent, commonly being manifest *in vitro* and *in vivo* at femtomolar concentrations (Fig 1.3). *In vitro* mutagenesis studies (Schor *et al.,* 2003; Millard *et al.,* 2007) indicate that MSF stimulation of fibroblast migration is mediated by two of its constituent IGD tri-peptide motifs located in structural modules 7FnI and 9FnI (Fig 1.2). The IGD motif is a highly conserved feature of fibronectin type I modules (Hynes, 1990), although no biological functionality had previously been ascribed to it. Significantly, synthetic tri- and tetra-peptides containing the IGD motif mimic all MSF bioactivities (Schor *et al.,* 1999; Schor *et al.,* 2003; Ellis *et al.,* 2010a). Initial data indicate that cellular response to MSF/IGD requires maintenance of integrin αvβ3 functionality and is mediated, at least in part, by the PI-3 kinase signal transduction pathway (Schor *et al.,* 1999; Ellis *et al.,* 2010b).

Full-length fibronectin isoforms do not express any of MSF's potent bioactivities, presumably as a consequence of steric hindrance of their constitutive bioactive motifs. The functional "unmasking" of these in MSF is accordingly postulated to result from appropriate alterations in its tertiary structure (higher order folding) resulting from truncation. Analogously, proteolytically-generated fragments of fibronectin display a host of "neo-activities" not expressed by the parental molecule (Fukai *et al.,* 1995), including the stimulation of monocyte migration (Clark *et al.,* 1988), the inhibition of cell proliferation (Muir and Manthorpe, 1992), the induction of protease gene expression by adherent synovial fibroblasts (Werb *et al.,* 1989), adipocyte differentiation (Fukai *et al.,* 1993) and an RGDS-independent mediation of cell migration (Fukai *et al.,* 1991). The two active IGD motifs in MSF (in I-7 and I-9) reside in the gelatin–binding domain (GBD), and as predicted, GBD generated by proteolytic cleavage of fibronectin, mimics all MSF bioactivities (Schor *et al.,* 1996, 2003). Interestingly, the proteolytically-generated cell-binding fragment

of fibronectin (containing the RGD motif) inhibits the motogenic activity of MSF, Gel-BD and IGD synthetic peptide (Schor *et al.,* 1999; unpublished observations).

Another bioactive motif has been identified in MSF. The putative zinc-binding motif (HEEGH), located in module I-8, is required for fibronectin-proteinase activity (Houard *et al.*, 2005). These workers further demonstrated that this motif is required to promote the migration of a breast tumour cell line (MCF-7). Mutagenesis of the two histidine residues to phenylalanine (FEEGF) abolished both the proteinase and motogenic activities of MSF.

Available evidence suggests that the motogenic response of target fibroblasts to MSF is mediated by its stimulation of HA synthesis: i.e. (i) exogenously provided HA stimulates the migration of adult-derived fibroblasts, and (ii) the motogenic response of adult fibroblasts to MSF is abrogated by co-incubation with Streptococcal hyaluronidase (Schor *et al.,* 1989). MSF is not unique in this regard, as other, but not all, motogenic cytokines also up-regulate HA synthesis which similarly appears to mediate cytokine-stimulated migration (Schor, 1994; Ellis *et al.,* 1992, 1997, 2007). As predicted, foetal fibroblasts produce significantly more HA (Chen *et al.,* 1989) and migrate to a greater extent compared to adult cells; hyaluronidase and MSF function-neutralising antibody reduces the elevated migration of foetal cells down to that of their adult counterparts. In contrast, endothelial cells, *in vitro,* do not synthesise HA (Winterbourne *et al*., 1983; Amanuma and Mitsui, 1991) either in the presence or absence of MSF (unpublished observations); the stimulation of endothelial cell migration and adoption of a sprouting cell phenotype by MSF *in vitro* must accordingly be mediated by an HA-independent mechanism. The situation *in vivo* is clearly more complex. In view of the well-documented effect of HA and HA fragments on angiogenesis, it is likely that the pro-angiogenic activity of MSF in animal model systems may also be indirectly mediated by its effect on HA synthesis by other cell populations (e.g. fibroblasts, carcinoma cells) within a multi-component tissue environment (unpublished data).

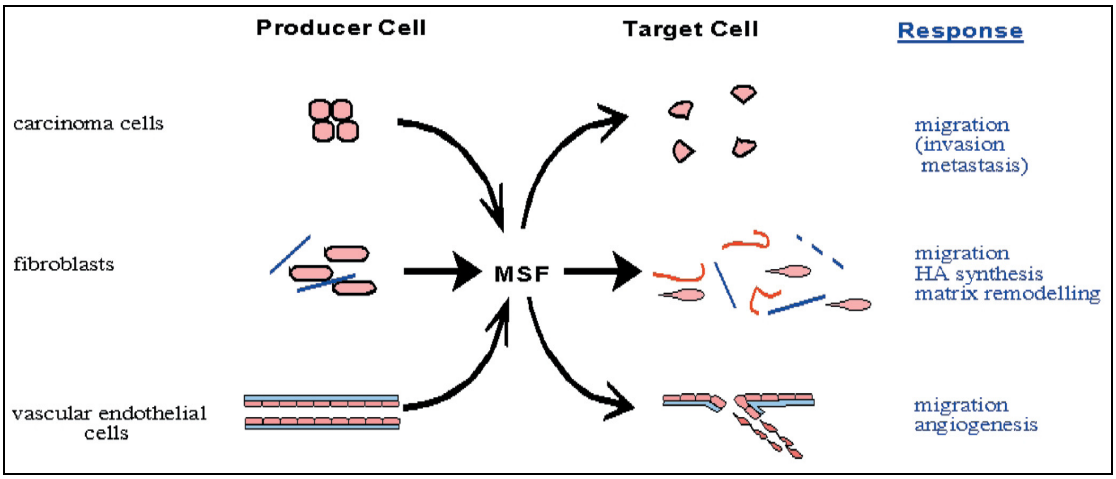


Figure 1.3: The expression of MSF bioactivity.
MSF secreted by three different cell line (producer cells) carcinoma cells, fibroblast cells and endothelial cells. The secreted MSF performs different bioactivities in the target cells in vitro and in vivo, including cell migration, HA synthesis, matrix remodelling and angiogenesis (adapted from Schor *et al.,* 2001).

1.2.3 MSF Expression

MSF exhibits an "oncofoetal" pattern of expression as defined by its (i) constitutive production by keratinocytes, fibroblasts and endothelial cells in foetal skin, (ii) reduced or undetectable expression in skin and other tissues in healthy adults, and (iii) re-expression by both carcinoma and stromal cells in the majority of common human tumours, including those of the breast, lung, colon, prostate (Schor *et al.,* 2003; unpublished observations). As is the case with other oncofoetal regulatory molecules, including full-length fibronectin isoforms containing the EDA and/or EDB type III modules (ffrench-Constant *et al.,* 1989).

Expression of MSF protein by adult skin fibroblasts is regulated by an unusual post-transcriptional mechanism involving the initial generation of a 5.9 kb MSF pre-message by read-through of the majority of the intron separating exons III-1a and III-1b of the fibronectin gene (Kay *et al.,* 2005). This precursor remains sequestered within the nucleus where it exhibits a short half-life. In response to an inter-dependent array of regulatory signals, the intron-derived 3'-UTR of the precursor mRNA is cleaved to produce the shorter 2.1 kb mature MSF message, which is then exported to the cytoplasm for translation (Kay *et al.,* 2005). This post-transcriptional control mechanism, coupled with the afore-mentioned message instability allows the cell to respond rapidly to micro-environmental cues, thereby controlling the temporal and spatial expression of MSF bioactivities. In contrast to this post-transcriptional

mechanism, epithelial and endothelial cells appear to constitutively express MSF protein and regulate the manifestation of its potent bioactivities by the variable co-expression of selective inhibitors.

In addition to its local expression by tumour cells and tumour-associated stromal cells, patients with cancer may also exhibit an aberrant systemic expression of MSF (Picardo *et al.,* 1991). For example, MSF is inappropriately expressed by skin fibroblasts obtained from distant uninvolved sites in patients with cancer; these aberrant "foetal-like" cells further differ from their normal adult counterparts in terms of their migratory phenotype and elevated expression of HA. Schor *et al.,* (1994) reported that the tumour-free, histologically normal, tissue margin adjacent to resected breast carcinomas commonly contain MSF-expressing intra-lobular fibroblasts (Schor *et al.,* 1994; unpublished observations). The presence of such a functionally disturbed peri-tumour field may carry as yet unrecognised prognostic significance, as well as provide useful insight into the factors (systemic and local) which contribute to cancer pathogenesis.

MSF bioactivity has also been detected systemically in the serum of approximately 90% of patients with breast cancer, as compared to only 10% of age- and sex-matched healthy controls (Picardo *et al.,* 1991; additional unpublished data). This systemic expression of MSF does not appear to be a measure of tumour burden (as is the case with other oncofetal tumour markers), as it may persist for decades after resection of the primary tumour in patients with no evidence of recurrent disease. The presence of bioactive MSF in the serum of cancer patients stands in marked contrast to results obtained in a corresponding study in patients recovering from post-surgical trauma; in these individuals, MSF bioactivity was not present in serum, in spite of being clearly detected locally in wound fluid (Picardo *et al.,* 1992).

MSF is present in wound fluid implicating a functional role in wound healing (Picardo *et al.,* 1992). MSF stimulated migration of high density adult fibroblasts in the 3D collagen gel assay (Schor *et al.,* 1988, 1993). MSF exerts a direct stimulatory effect upon the synthesis of high weight hyaluronic acid (HA) and it is the accumulation of this HA in the matrix which is responsible for the observed increase in migrating activity (Schor *et al.,* 1989). The influx of fibroblasts into the wound site and the transient elevation in HA level in granulation tissue are both consistent with a suggested role in wound repair. TGF-β1 is a potent antagonist of MSF, blocking its

stimulation of fibroblast migration and HA synthesis (Ellis *et al.,* 1992), and might thereby regulate MSF activity in the wound.

1.2.4 Inhibitors of MSF activity

1.2.4.1 Neutrophil gelatinase-asscciated lipocalin (NGAL):

NGAL (Lipocalin-2), belongs to a family of low molecular weight proteins, is an inhibitor of MSF motogenic activity. NGAL is a 25kDa protein which was first identified in neutrophils, hence its name NGAL. Jones *et al.,* (2007) studied the bioactivity of MSF in keratinocytes. They compared the migration activity of tumour derived fibroblasts and the paired forearm skin fibroblasts of cancer patients with the keratinocytes cell line (HaCat) in collagen migration assay. They found that the conditioned medium of early passage keratinocyte or a keratinocyte cell line (HaCat) did not show any migration stimulating activity. This result led to the hypothesis that even though keratinocytes secrete MSF, the co-secretion of a functional inhibitor masks its bioactivity, whilst the tumour derived and the paired forearm skin fibroblasts exhibit an effective bioactivity. So a putative inhibitor (MSFI) can regulate the bioactivity of endogenous MSF and its play an important role in the inhibition of MSF induced migration activity. Fractionated keratinocyte conditioned medium by size-exclusion chromatography revealed the presence of bioactive MSF and inhibitor of MSF (MSFI) with size of 70kDa and 25kDa respectively. This inhibitor of MSF activity was isolated and identified as NGAL (Lipocalin-2). Expression of MSF and NGAL was confirmed in keratinocyte cells by immunostaining studies (Jones *et al.,* 2007). Experiments in the collagen gel assay also reported that NGAL dose not affect the viability or proliferation of the target cells.

NGAL is a multi-functional protein, performing a wide range of mechanisms. NGAL can bind ferric siderophores which enables it to perform a wide range of functions including iron transport, controlling iron-responsive genes and also to act as bacteriostatic agent. NGAL is vital during mesenchymal-epithelial transition and also in Ras-transformed mesenchymal tumour cells. Even though the iron-chelator Dimethyl sulphoxide (DFOM) is capable of reversing iron dependant NGAL activity, it does not affect its inhibitory affect on MSF, suggesting iron is not involved in this process (Jones *et al.,* 2007).

1.2.4.2 Endothelial MSF-Inhibitor (eMSFI)

Endothelial cells produce a wide range of cytokines, extracellular components and cell surface molecules. Different cytokines produced by endothelial cells include interleukin 1, 6, and 8, platelet derived growth facor (PDGF), Endothelin, basic fibroblast growth factor (bFGF), insulin like growth factor and TGF-β1. Molecules secreted by endothelial cells include prostacyclin, nitric oxide, platelet activating factor and plasminogen activator inhibitors-1 etc. Many of the soluble factors and extracellular matrix components synthesised by endothelial cells can control fibroblast migration and matrix biosynthesis in processes such as angiogenesis and wound healing i.e. TGF-β1 and PDGF. Endothelial cells *in vitro* show two reversible phenotypes namely cobblestone and sprouting (Schor and Schor, 2010). These cells in culture form a cobblestone monolayer on the surface of two dimensional (2D) substrata, whereas the same cells show elongated sprouting morphology when embedded within three dimensional (3D) collagen gels (Sutton *et al.,* 1991). Conditioned media (CM) collected from cobblestone endothelial cells inhibits MSF stimulated cell migration, whereas CM collected form sprouting endothelial cells shows normal MSF like activity (Schor and Schor, 2010). The above observations indicate the presence of an inhibitor of MSF activity (eMSFI) in cobblestone CM which has been identified as *Insulin-like growth factor binding protein-7* (IGFBP7) by (Dr Sarah Jones/ Cell and Molecular Biology Unit/ Dental School). She purified IGFBP7 from Endo 742 CM by diethylaminoethyl cellulose (DEAE) chromatography (Jones *et al.,* unpublished data) and gel elution (unpublished data). Insulin-like growth factor binding protein-7 (IGFBP7), also known as mac25 or IGFBP-related protein-1 (IGFBP-rP1) is a secreted protein belonging to the IGFBP family. The major function of the protein is the regulation of availability of Insulin like growth factor (IGFs) in tissue as well as in modulating IGF binding to its receptors (Oh *et al.,* 1996; Pollak, 2008). Oh *et al.,* (1996) shown that IGFBP-7 has a wider distribution in normal tissue and has lower expression in several cancer cells (breast, prostate, colon and lung) indicating that IGFBP-7 may function as a growth-suppressing factor, as well as an IGF-binding protein.

1.2.4.3 Transforming Growth Factor-β1

Transforming growth factor type β1 (TGF-β1) is a homodimeric polypeptide of molecular weight 25kDa that belongs to a large family of multifunctional secreted homologous proteins. This family of polypeptides includes the TGF-β 1-5 and a number of other similar proteins.

TGF-β1 participates in a number of cellular mechanisms and is a potent regulator of cell growth, differentiation, degradation of matrix proteins such as collagen and fibronectin, hematopoiesis, mammary gland development, bone metabolism, skin formation, angiogenesis, inflammation and repair**,** chemotoxins, and fibroblast proliferation (Robert et *al.,* 1990). TGF-β actions and mechanism vary from cell to cell according to different parameters including type of target cell and the conditioned media. TGF-β has a pleiotropic effect and may have a stimulatory or inhibitory effect on cell migration, proliferation, protein synthesis and differentiation. Levy *et al*., 2006 data support TGF-β's role as an important component in the initiation and progression of cancer. TGF-β has a dual role in tumourigenesis;
In the initial stages TGF-β acts as a tumour suppressor and inhibits growth of epithelial cells, endothelial cells and early cancer cells. Whereas later on, TGF-β accelerates the malignant process enhancing survival, progression and metastasis of the tumour in an established tumour by promoting angiogenesis and escape from immune surveillance (Rangnathan *et al.,* 2007). These observations indicate that the normal epithelial cells behave differently than cancer cells in response to TGF-β. Tumour cells and normal cells show different gene expression in response to TGF-β. For example tumour cells show increase in the production of proteases and down-regulation of the inhibitors of proteases in response to TGF-β, whereas this is not observed with normal cells. The mechanism responsible for this differential response of various cell types is not clear.

The cytokine TGF-β1 acts as a potent inhibitor of fibroblast cell migration and HA synthesis. HA synthesis stimulation by TGF-β1 is largely based on the anatomic site, for example, TGF-β1 can promote HA synthesis in foetal lung fibroblasts but not in foetal skin cells (Ellis *et al.,* 1995). Ellis *et al.,* (1992) studied the antagonistic effect of TGF-β1 on MSF, the study revealed that TGF-β1 blocks the migration of sub-confluent adult fibroblasts and high molecular weight HA synthesis. TGF-β1 is a potent inhibitor of MSF-stimulated cell migration.

1.3 Aims of the study

In this thesis I studied the possible role of migration stimulating factor (MSF) on the progression of oral tumour, focussing on oral squamous cell carcinoma (OSCC) and salivary gland tumours (SGT). The overall objectives of this study were:

1- To ascertain the possible presence, diagnostic and prognostic value of MSF in OSCC and SGT.
2- To determine the effects of MSF on the migration of oral tumour cell lines and normal stromal cells.
3- To identify the putative MSF receptors.

The specific studies carried out towards these objectives included:

1. To ascertain the possible presence, diagnostic significance and role of both MSF isoforms in SGT. Immunohistochemical was used to compare the expression pattern of MSF in histologically normal salivary gland tissue, benign and malignant SGT.
2. To determine the diagnostic and prognostic value of MSF expression in OSCC.
3. Determine the effect of MSF on different types of cells (TYS, HSG, Endo 742 and FSF44) in terms of migration.
4. Determine the specific amino acid motifs of MSF (IGD and/or HEEGH or both) that are responsible for its bioactivity.
5. Determine the modulation of MSF bioactivity by antibodies (PEPQ, TYN and anti-αvB3 Ab) and other proteins (NGAL and IGFBP-7).
6. Determine the expression of MSF by oral tumour cell lines.
7. To verify the receptors responsible for the motogenic activity of MSF.

2. Chapter two: Materials and Methods

2.1 Bacterial rhMSF WT Preparation

The following recombinant proteins have been purified for use in this study:
- rhMSF+aaWT; rhMSF-aaWT; rhMSF+aaDGI 3, 5 and rhNGAL. The purification method was identical for all recombinant proteins and rhMSF is given as an example.

2.1.1 Expression of recombinant MSF in Bacteria

Background:

The gene encoding MSF was cloned into the expression vector pRSET. When the recombinant protein was expressed using this plasmid, it had two N-terminal tags; a poly-histidine tag (his6) to aid subsequent purification and the Xpress tag, which is recognised by a commercially available antibody (Invitrogen, Paisley, UK).

Materials:

Plasmid pRSET (Invitrogen, Paisley, and UK); L-broth medium; 50µg/ml carbenicillin; Isopropyl-β-D-galactopyranoside (IPTG) and competent DE3 plysS cells.

Table 2.1: Protein and its plasmid encode

Protein name	Plasmid name
rhMSF+aa WT	pRSET MSF+aa
rhMSF-aa WT	pBEN0404 MSF-aa
rhMSF+aa DGI 3,5	pRSET MSFDGI 3,5
rhNGAL	pRSET LCN2

Methodology:

The plasmid pRSET containing the MSF gene (Table 2.1) was transformed into competent DE3 plysS cells. The cells were plated on LB-agar containing 50µg/ml carbenicillin and then incubated at 37°C overnight.

A single bacterial colony from the plate was then used to inoculate 10ml of L-broth containing 1% (w/v) glucose and 50µg/ml carbenicillin. This culture was then grown at 37°C overnight, in an orbital shaker (≈275rpm). The overnight culture was then used to inoculate 2 x 100ml cultures of L-broth containing 1% (w/v) glucose and 50µg/ml carbenicillin. These cultures were grown in the same conditions, to an optical density at 600nm (O.D. 600nm) of between 0.4 and 0.6. Transcription and translation

i.e. protein synthesis was then induced by addition of IPTG to a final concentration of 1mM. Induction was then allowed to continue at 37°C for 3-5 hours in the orbital shaker (incubator). The bacteria were then pelleted by centrifugation at 3000rpm for 10 minutes at 4°C. The supernatant was then removed and collected for sterilisation and the bacterial pellets were then stored at -20°C.

2.1.2 Extraction of recombinant protein from bacterial cell pellets

Materials Required:

Bacterial pellets (Bacterial cell pellets collected after induction and expression of rhMSF); BugBuster (Invitrogen); Benzonase (Invitrogen); 1M Dithiotrietol (DTT); 500mM CAPS pH 11; 20mM Tris-HCl pH 8.5; 30% (w/v) N-lauryl sarcosine and 10mg/ml lysozyme.

Methodology:

The recombinant protein is present as insoluble inclusion bodies when expressed in bacteria.

Each bacterial pellet, collected from 50ml culture was resuspended in 2.5ml of BugBuster with the addition of 1µl/ml benzonase. The pellet was thoroughly resuspended by vortexing and then the tubes were shaken vigorously for 20 minutes. The bacterial cell lysate was pooled and split into 5ml aliquots and centrifuged at 20,000rpm for 15 minutes (Hereaus). The supernatant containing the soluble bacterial proteins was then collected and frozen at -20ºC. Lysozyme was then diluted 50x with BugBuster and 5ml added to each insoluble protein pellet. The pellet was then thoroughly resuspended and incubated at room temperature for 20 minutes, with gentle agitation, then 30ml of 1/10 dilution BugBuster was added to each tube spun at 20,000 rpm for 20 minutes, the supernatant was then removed completely and discarded each pellet was then resuspended and washed for a second time. Each pellet was then dissolved in 2.5ml of 500mM CAPS pH 11. 0.5% (w/v) n-laurylsarcosine, 10mM DTT for up to 1 hour at room temperature then spun at 20,000 rpm for 10 minutes. The solubilised protein was then dialysed (Pierce dialysis cassette 3.5kDa cutoff) using 2 litres of 20mM Tris-HCl pH 8.5 0.1mM DTT (4 hours at 4ºC) the buffer was then replaced and dialysis continued overnight, finally the protein was

dialysed for four hours at 4ºC in 2 litres 20mM Tris-HCl pH 8.5. The protein (rhMSF) was then purified on a HiTrap (Pharmacia) Chelating column, charged with nickel.

2.1.3 Bacterial rhMSF Purification

Materials:

Washing buffer: 20mM sodium phosphate (NaH_2PO_4) and 0.5M sodium chloride (NaCl).

Elution buffer: washing buffer + 0.5M imidazole. Both buffers were adjusted to pH 7.6 – 8.0 using NaOH.

Overview:

After the final dialysis step, the recombinant MSF was purified from the protein extract by affinity chromatography. The affinity column (HiTrap) was charged with nickel sulphate and the rhMSF had been engineered to have an N-terminal tag containing 6 histidine residues which can bind to nickel. The dialysed extract was pumped onto the column, which was then washed to remove unbound material and the rhMSF eluted by an increasing gradient of imidazole which competes with the rhMSF to bind to the column.

2.1.3.1 Charging the HiTrap Chelating Column (1ml) With Nickel

The Hi-Trap column was washed with 5ml of distilled water (to wash away the 20% ethanol storage solution)**.** Then 500µl of 0.1M Nickel Chloride was then applied**.** The HiTrap column was then washed with 10ml of distilled water

2.1.3.2 Affinity Purification of rhMSF

Materials:

Affinity column charged with 0.1M Nickel chloride.

Column Used: Hi-Trap chelating 1ml (GE Healthcare)

Reagents used:

Binding buffer: 20mM sodium phosphate, 0.5M sodium chloride, pH 7.6 – pH 8.0.

Eluted buffer: as binding buffer + 500mM imidazole.

Methodology:

The nickel charged column was equilibrated with 10 column volumes (cv) of binding buffer at a flow rate of 1ml/minute. The dialysed crude extract containing rhMSF was spun at 13,000 rpm for 5 minutes. The sample was then applied to the column at a slower flow rate of 0.3ml/minute. Fractions were then collected during sample application and column washing. The column was then washed again (1ml/minute) with binding buffer to remove any unbound proteins. The rhMSF was then eluted from the column by running a 0-500mM imidazole gradient through the column over 15cv. The rhMSF usually eluted at around 250mM imidazole. 1ml fractions were collected during the gradient phase. The presence of rMSF was confirmed by SDS PAGE or Western blotting, where the protein runs at a molecular weight of ~77kDa under reducing conditions.

2.1.4 Endotoxin treatment

Endotoxin also called lipopolysaccharides (LPS) is a natural component of the cell wall of most gram- negative bacterial. Bacterial expression systems are widely used to produce different types of proteins and therefore these samples may be contaminated with endotoxin. In order to remove endotoxin from recombinant protein preparations different processes have been developed, these include two-phase extractions, ultrafiltration, hydrophobic interaction chromatography, ion exchange chromatography.

2.1.4.1 Two-phase extraction:

Triton X-114 (Sigma) was added to the protein solution to a final concentration of 1% (v/v) in a sterile microcentrifuge tube (pyrogen free). The mixture was incubated at 4°C for 45-60 minutes with end over end mixing to ensure a homogenous solution. The sample was then transferred to a 37°C water bath and incubated for 15-60 minutes until the solution appeared cloudy. The mixture was then centrifuged for 10 minutes at 13000 rpm at 37°C. The upper layer containing the protein (endotoxin-free) was carefully removed from the tubes and pooled in a sterile tube in order to test with LAL kinetic chromogenic assay to determine endotoxin units per millilitre (EU/ml).

2.1.4.2 The Limulus Amebocyte Lysate (LAL) assay:

Endotoxin levels were measured by LAL assay. The reagent preparation and experimental protocol used was that supplied with the kit. Different dilutions of the protein and buffer samples were prepared using the LAL reagent water as the diluent. Positive controls containing endotoxin standards and negative control containing the LAL reagent water were run at the same time. After incubation and in the presence of endotoxin, gelation occurs. The test is positive if a gel has formed and remains intact in the bottom of the tube after inversion, whilst in the absence of endotoxin, gelation does not occur, the test is considered negative. After removed of the endotoxin from the purified proteins, all the proteins purified in this study were dialysed against PBS except rhNGAL which was dialysed using 20mM sodium phosphate, 500mM sodium chloride, pH 7.6 – pH 8.0.

2.2 Identification of protein

2.2.1. SDS-PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis)

Materials:

1M Tris HCl pH 8.7 and pH 6.9 (Sigma); 30% Acrylamide/Bis-solution 37.5:1 (2.6% c) (Bio-Rad); 10 % (w/v) SDS(Melford); APS ammonium persulphate (Bio-Rad); TEMED(Sigma); Laemmli loading buffer (Bio-Rad Laboratories Ltd,. Hemel Hempstead, Hertfordshire UK); Magic Markers (Invitrogen Ltd, Paisley UK) Molecular weight markers (Bio-Rad, Glycine (Sigma); TGS buffer: Glycine 1.92M; SDS 1 % (w/v); Tris 250mM, 2-mercaptoethanol; Gel CodeBlue (Pierce).

Methodology:

The purified protein quality was analysed using SDS-PAGE which separates proteins according to their molecular weight under an electrical current. The pH difference was established by the resolving and stacking gel. SDS PAGE was carried out according to the method of Laemmli (1970). Typically 10% separating gels with 4.5% stacking gels were run, under reducing conditions. Samples were made up in an equal volume of Laemmli loading buffer containing 5% (v/v) 2-mercaptoethanol and

heated for 5-10 minutes at 95˚C prior to loading onto the gel. Precision protein plus molecular weight markers were also loaded. Magic Markers (Invitrogen Ltd, Paisley UK) were loaded for molecular weight estimation, on gels that were to be subsequently Western blotted. Electrophoresis buffer was 1 x TGS (Tris Glycine SDS, Bio-Rad). The gel was then run at 150-180v until the dye front reached the end of the gel. Lanes containing molecular weight markers and samples for sequencing were washed 3 x 5 minutes in distilled water and then stained in Gel Code Blue (Perbio).

2.2.2 Western blots:

Western blotting is a frequently used method to detect specific proteins in a complex mixture of samples like body fluids or cells. In this technique SDS polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate the protein by size, and then the protein bands are transferred from the gel onto a membrane such as nitrocellulose or PVDF using an electric current. The technique is dependant on the use of a specific antibody directed against a required protein after blocking the non-specific binding sites on the blot by using blocking buffer. After washing unbound primary antibody the secondary antibody is added which can only recognise this protein primary antibody and is conjugated with the enzyme horse radish peroxidase (HRP) for visualisation. The conjunction of enzyme (HRP) with a chemilumienscent substrate can be visualised to determine the location of the antibody.

Materials:

- Transfer Buffer: 5.82g Tris; 2.93g Glycine; 3.75ml 10% (w/v) SDS; 200ml Methanol made up to1litre with dH_2O.
- TBST: 24.2g Tris; 80g Sodium chloride; 5ml Tween; 14ml Hydrochloric acid pH 7.6; 1litre dH_2O.
- Blocking Buffer; 100ml 1X Tris-buffered saline Tween TBST (PH 7.6); 1g dried milk powder.
- TBS: TBST without Tween 20
- Bio Rad Extra thick blotting paper.
- Nitrocellulose membrane (0.2 μm; Bio-Rad)
- Bio-Rad's Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell
- Super signal West Dura (Pierce) (Enhanced Chemiluminescence substrate)

Antibodies:
Primary antibodies:

- Rabbit polyclonal identification antibody RpVSI (Stock Conc 20µg/ml, 2005), 2µg/ml in 1% (w/v) BSA-PBS-T. (Working concentration)

- Goat Anti- human integrin αV lCD 51 antibody,R&D Systems cat no. AF1219 (Stock Conc 200µg/ml), 200ng/ml in 1% (w/v) Milk TBST.

- Anti- human integrin β3 (CD61) Mouse Monoclonal antibody,R&D Systems cat no. MAB2266, (Stock Conc 500µg/ml), 2µg/ml in 1% (w/v) Milk TBST

Secondary antibody:

- DAKO rabbit anti-goat HRP was used at a 1:10,000 diluted in 1% (w/v) Milk TBST.
- DAKO rabbit anti-mouse HRP was used at a 1:10,000 diluted in 1% (w/v) Milk TBST.
- DAKO Goat anti- rabbit HRP was used at a 1:10,000 diluted in 1% (w/v) Milk TBST.

Methodology:

The purified protein quality was analysed using SDS-PAGE which separates proteins according to their molecular weight under an electrical current. The pH difference was established by the resolving and stacking gel. A 10 % acrylamide resolving gel was used for the rhMSF (as example of purified protein had been used in this study). The wells were created in the stacking gel which stacks the proteins, which then passes through the resolving gel where the separation of the protein takes place. The samples and markers were prepared in Laemmli loading buffer containing 2-mercaptoethanol and were heated for 5 minutes at 95 ºC to denature the proteins for effective separation. When the gel kit was ready it was suspended in the gel tank in TGS buffer the gel was allowed to run by providing 180V, 150 W and 20mA until the dye front reached the bottom of the gel. The gel was stained with GelCode blue solution; until desired stain was obtained and destained with water.

The bands thus obtained were analysed for quality analysis where the marker provides the identification of protein molecular weight. The protein gels that obtained from the SDS-PAGE were used for semi-dry Western blotting. The gels were trimmed to size and then rinsed in the transfer buffer. The Western blot was prepared by

placing pieces of BioRad extra thick paper soaked in transfer buffer onto the anode, followed by nitrocellulose membrane, gel and again another piece of soaked BioRad extra thick paper on top. The proteins were blotted onto the membrane at 15V for 42 minutes. The proteins were blocked by using 1 % (w/v) dry milk in TBST (Tris buffer saline containing 0.05% Tween-20). The membrane was then probed overnight with primary antibody, excess unbound primary antibody was then removed by washing with TBST. The membrane was then probed with the HRP-labelled secondary antibody in TBST for 60 minutes. After removing the excess secondary antibody by washing with TBST, the secondary antibody was localised by reaction with Pierce Supersignal for 1-5 minutes. Chemiluminescence was visualised and documented using the GeneGnome (Syngene).

2.2.3 Dot Blots.

Samples of rhMSF +aa and Fn were concentrated by series dilutions of 100ng/ml, 200, 400, 800ng/ml and 500ng/ml, 1 µg/ml, 2µg/ml, 4µg/ml for both samples respectively. For dot blots, nitrocellulose sheets were cut to an appropriate size, marked into 1cm squares, spotted with the test protein solutions and allowed to air dry. The blots were then blocked with TBST plus 1% (w/v) Marvel dried milk powder for 20 minutes at room temperature. Blots were then incubated with the appropriate antibody overnight, rinsed three times with TBST Tween 20, incubated with HRP conjugated secondary antibody for 2 hours, washed three times in TBST at room temperature, and finally washed for 5 minutes with TBS. Visualisation was achieved by enhanced chemiluminescence using SuperSignal (Pierce) and the GeneGnome (Syngene).

2.2.4 ELISA (enzyme-linked immuno-sorbent assay)

This method makes used of an enzyme that reacts with a colourless substrate to produce a coloured product. The enzyme is covalently linked to a specific antibody that recognises a target antigen. ELISA is a simple and highly sensitive method of analysis that allows for simultaneous and rapid quantification of a large number of samples.

2.2.4.1 Materials

ELISA plates Colorimetric assay: 96-well plates (polystyrene, treated surface, flat bottom), [Corning-Costar, cat. no. 07-200-38 (9018).]

Standard proteins and other routinely tested antigens

- Recombinant human MSF (rhMSF) and (rhMSF-aa) prepared in house, of known concentration, was used as a standard protein for quantification of MSF concentration in analysed samples.
- Human cellular Fn [Upstate, cat. no. 08-102]. A 45kDa proteolytic fragment of the human plasma Fn containing a gel-binding domain (GBD) [SIGMA, cat. no. F0162]

Buffers:

- Washing buffers:

1x PBS - prepared from tablets (Phosphate buffered saline. SIGMA, cat. no. P-4417) according to manufacturer instructions, filtered.

1x PBST-1x PBS with final conc 0.05% Tween 20 (10% Tween Surfact-Amps 20. Pierce, cat. No. 28320), filtered.

- Coating buffer: Commercial Carbonate-Bicarbonate buffer (Pierce, cat. no. 28382)
- Blocking buffers: 1% (w/v) bovine serum albumin (BSA), ELISA grade (98% purity) (SIGMA, cat. No. A7030) in PBST.

Antibodies:

Primary antibodies

- RpVSI, 2μg/ml in 1% BSA-PBS-T.
- Anti-cell binding domain of Fibronectin antibody (CHEMICON, cat. no. MAB1937)
- TYN 1.1 (batch 3) –Mouse monoclonal antibody to MSF- aa, purified on protein L. prepared in house.

- PEPQ 1.2**:** (mouse monoclonal anti-IGD), Stock concentration 61.4 µg/ml, Batch no.2, prepared in house.
- Anti-human NGAL (goat polyclonal AF1757), (R&D systems)
- Recombinant αvβ3 (R&D Systems Cat. # 3050-AV-050) (Lot # OMM0409081)

Secondary antibodies

- Goat anti-rabbit conjugated with horse radish peroxidase (GAR-HRP), (DAKO or Pierce)
- Rabbit anti-mouse conjugated with horse radish peroxidase (RAM-HRP), (DAKO)
- Rabbit anti-goat conjugated with horse radish peroxidase (RAG-HRP), (DAKO)

Substrate for HRP: TMB [TMB^+ Substrate Chromogen, DAKO, cat. no. S1599]

ELISA plate reader: ELISA plate reader (MRX Dynex) was used to measure resultant optical density of the colorimetric assay. The reader was run under computer control with software supplemented by the manufacturer (REVELATION©, version 3.2). The program for MSF quantification was created in the Revelation software as an endpoint measurement with following parameters:

- Dual wavelength mode (filters 450/570nm).
-Template (96-well plate) setting – position of blanks, standards and test samples - was defined manually before each measurement.
- Blanking was performed automatically by subtracting the averaged blank values in a given plate from all standards and test sample values.

2.2.4.2 Methodology

2.2.4.2.1 Indirect ELISA:

Polystyrene 96-well microplates (Costar) were coated overnight with rhMSF-aa, rhMSF +aa, cellular fibronectin (cFN) and fibronectin gel binding domain (GBD) diluted in carbonate buffer pH 9.6. After washing with PBS, non-specific binding sites were blocked by incubation with 200µl/well 1% (w/v) BSA in PBST (BSA-PBST) for 1 hour at room temperature with gentle agitation. After further washing, 100µl/well TYN 1.1, RpVSI antibodies were added diluted to 1µg/ml and 10µg/ml respectively

in BSA-PBST and the plate was then incubated for 1 hour at ambient temperature. Antibody binding was detected by incubating the plate for 1 hour at ambient temperature with 100µl/well of HRP -conjugated rabbit anti-mouse secondary antibody diluted 1:1000 in BSA-PBST, followed by further washing and incubation with tetramethylbenzidine (TMB), (Code S1599 from DAKO UK Ltd., Ely, Cambridgeshire UK), for 10 minutes with gentle orbital shaking. The reaction was stopped by adding 50µl/well 2M H_2SO_4, and the OD at 450 nm was measured using a plate reader (MRX DYNEX Technologies Limited, Worthing. West Sussex UK).

2.2.4.2.2 Sandwich ELISA:

Polystyrene 96-well microplates (Costar, Insight Biotechnology Limited, Wembley, UK) were coated overnight at room temperature with 1µg/ml of MAB PEPQ 1.2 as the capture antibody, 100µl/well diluted in coated buffer. After washing with PBS, non-specific binding sites were blocked by incubation with 200µl/well 1% (w/v) BSA in PBST (BSA-PBST) for 1 hour at room temperature with gentle agitation. The plate was then washed with PBS, twice with PBST and again with PBS. Recombinant MSF was plated 100µ/well and incubated for 1 hour at room temperature. After washing with PBS, twice with PBST and again with PBS, 100µl/well RpVSI diluted to 10µg/ml in 1% (w/v) BSA-PBST was added and then the plate was incubated for 1 hour at ambient temperature. Followed washing as described above, goat anti-rabbit (Pierce) HRP conjugate was then added to each well at a dilution of 1:1000 and incubated for one hour at room temperature, followed by the washing procedure, tetramethylbenzidine (TMB), (Code S1599 from DAKO UK Ltd., Ely, Cambridgeshire UK), was added 50µl/well and left for 10 minutes with gentle orbital shaking. The reaction was stopped by adding 50µl/well 2M H_2SO_4, and the OD at 450 nm was measured using a plate reader (MRX DYNEX Technologies Limited, Worthing. West Sussex, UK).

2.2.5 Electro-Chemiluminescenes detection (Meso Scal Discovery Technology MSD)

Another ELISA technique was used in this study that was more sensitive than the basic one and required less volume of sample this was called MSD electrochemiluminescence.

The MSD electrochemiluminescence detection used SULFO-TAG™ labels that emit light upon electrochemical stimulation initiate at electrode surfaces of MULTI-ARRAY and MULTI-SPOT microplates. In Electro-Chemiluminescence a reaction cascade transfers electrical energy from the plate surface to a light-emitting antibody / transition metal complex.

The Sector Imager 6000 uses an ultra-low noise charge-coupled device (CCD) camera with custom-designed telecentric lenses for rapid detection in all MULTI-ARRAY® or MULTI-SPOT® plates.

Materials:

- **MSD has combined MULTI-ARRAY ® microplate:** 96 multispot plate using direct immobilised on uncoated standard surface.

- MSD® Read Buffers T 4x

Antibodies:

Identification Antibody

- Rabbit polyclonal identification antibody RpVSI, prepared in house (stock Conc 20µg/ml, 2005), 2µg/ml in 1% (w/v) BSA-PBS-T.

- Anti- human integrin αVβ3 antibody Mouse MAB3050 (R&D Systems lot # VUK02). Stock Conc of 500µg/ml.

- Anti- human integrin αVlCD 51 antibody Goat AF 1219 (Stock Conc 200µg/ml), 2µg/ml in 1% BSA-PBS-T

- Anti-human integrin β3 CD61 Mouse Monoclonal Antibody (Stock Conc 500µg/ml), 2µg/ml in 1% BSA-PBST

- Anti- human lipocalin/ NGAL Goat Polyclonal AF1757 (Stock Conc 200µg/ml). 2µg/ml in 1% (w/v) BSA PBST.

- IGFBP7 antibody (R&D Systems Polyclonal Goat IgG.Catalog # AF1334. lot # C0108051). Stock Conc of 100µg/ml, 1µg/ml in 1% (w/v) BSA-PBST.

- TYN 1.2 Mouse monoclonal antibody to MSF aa-, purified on protein L. prepared in house (Stock Conc 100µg/ml).

Anti-Species Antibody:

- MSD Sulph-Tag Goat -anti- Mouse ab (Cat. No. R32AC-1), (Stock Conc 500µg/ml). 2µg/ml 1% (w/v) BSA- PBST.
- MSD Sulph-Tag Goat -anti- Rabbit ab (Cat. No. R32AB-5), (Stock Conc 500µg/ml). 2µg/ml in 1% (w/v) BSA- PBST.
- MSD Sulph-Tag Donkey -anti-Goat ab (Cat. No. R32AG-5), (Stock Conc 500µg/ml). 2µg/ml in 1% (w/v) BSA- PBST.

Methodology:

Indirect MSD ELISA:

Samples were plated 25µl/well and incubated overnight at 4°C (tapping the plate after addition to allow the liquid to coat the bottom of the well). Wells were then washed once with PBS and twice with PBST (0.05% Tween), then 150µl/well of blocking buffer 1 % (w/v) BSA- PBST was added and incubated for 1hour with shaking. After washing with 150µl PBST per well, 25µl of detection antibody was added and the plate was then incubated for two hours at room temperature with shaking. Followed washing as described above, 25µl of anti-species antibody (2µg/ml) was added and incubated with shaking for 1 hour at ambient temperature, followed by the washing procedure. 150µl 2X Read buffer was then added to the wells before reading (1 part 4x Read Buffer: 1 part distilled water) and then the plate was read by the Sector Imager 6000 reader.

Sandwich MSD ELISA:

96-well plates were coated overnight at 4ºC with the capture antibody, 30µl per well diluted in PBS. The plate was then washed (3x) with PBST, 150µl per well. The plate was then blocked under constant orbital shaking for 1 hour at room temperature with 5% (w/v) BSA PBS (150µl per well). The plate was then washed with PBST (3x), 150µl per well and samples were added 25µl per well with constant orbital shaking for two hours at room temperature. After washing as described above, 25µl per well of detection antibody in 1 % (w/v) BSA PBST was added with constant shaking for 2 hours at room temperature. The plate was then washed as above, and incubated with Anti-Species Antibody: MSD Sulph-Tag in 1% (w/v) BSA PBST. 25µl per well, with orbital shaking for 2 hours at room temperature followed by the washing procedure. 150µl 2X Read buffer was then added to the wells before reading

(1 part 4X Read Buffer: 1 part distilled water) and then the plate was read by the Sector Imager 6000 reader.

2.3 Affinity purification

2.3.1 Purification of IgG from Mouse Monoclonal Antibodies

Monoclonal antibodies were purified from large volumes of hybridoma supernatant using Hitrap Protein G columns.

Materials:

- Dialysis buffer: 20mM Sodium phosphate pH 7.0.
- Binding buffer: 20mM Sodium phosphate pH 7.0.
- Elution buffer: 0.1M Glycine pH 2.7
- Antibody storage: 50µl1M Tris-HCl pH 8.0 added to the eluted sample per ml
- 20% (v/v) Ethanol in distilled water (1 litre) for column storage and BioCAD

Methods

Conditioned media (CM) was dialysed using Pierce Snakeskin dialysis tubing against 20mM Tris HCl pH 7.0 2 x 4 litres overnight at 4°C, the buffer was changed after the first hour. Then sample was spun at 12.000rpm for 10 minutes to remove particulates before chromatography. The HiTrap protein G column was then equilibrated with binding buffer. The sample was then pumped on slowly to facilitate maximum binding and the column was then washed with binding buffer to remove any unbound material. The buffer was then changed to elution buffer and the low pH caused antibody elution. The low pH was not suitable for antibody storage so the pH was raised by addition of 1M Tris-HCl pH 8.0 (50µl/ml) to the test tubes either before the experiment or immediately after the antibody elutes. The concentration of antibody was estimated by absorbance at 280nm on the spectrophotometer. The protein concentration of the antibody was determined by the following equation:

$$\text{Conc. mg/ml} = \frac{\text{OD 280nm} \times 10 \times \text{dil. Factor}}{14}$$

The purified antibody was aliquotted and stored at -20°C. The column was then re-equilibrated with binding buffer and then washed with 20% ethanol for storage.

2.3.2 Purification of IgG from Rabbit Polyclonal Antibodies

Immunised rabbit serum was heat inactivated and the IgG was purified using HiTrap Protein A columns

Materials:

- Dialysis buffer: 20mM Sodium phosphate pH 7.0.
- Binding buffer: 20mM Sodium phosphate pH 7.0.
- Elution buffer: 0.1M Glycine pH 2.7
- Antibody storage: 50µl1M Tris-HCl pH 8.0 added to the eluted sample per ml
- 20% (v/v) Ethanol in distilled water (1 litre) for column storage and BioCAD

Methods:

After thawing, the serum was heat inactivated by heating to 56ºC for 30 minutes. The serum was then dialysed using a Pierce cassette (3.5kDa cut off) against 20mM Tris HCl pH 7.0 2 x 4 litres overnight at 4°C, the buffer was changed after the first hour. The sample was then spun at 12.000rpm for 10 minutes to remove particulates before chromatography. The Protein A column was then equilibrated with binding buffer. The antibody was then purified following the previously described method on page (63).

2.3.3 Affinity column preparation

Several affinity columns were prepared for use in this study using different proteins and antibodies as mentioned below:

2.3.3.1 Materials

A- Reactigel resin (Pierce CDI- Agarose)

B- Buffer:

Coupling buffer: 100mM borate made up to a pH 10.

Blocking buffer: 50mM Tris HCl pH 10

Wash buffer: phosphate buffered saline (PBS).

C- Proteins used in this study for affinity column preparation:

1- 10µg of procaryotic recombinant MSF WT (rhMSF WT +aa) was dialysed against 50mM sodium phosphate (NaH_2PO_4) + 150 mM NaCl pH 8.0.using Pierce dialysis cassettes (cut-off 3.5kDa), overnight. The sample was then coupled to the activated resin then continued overnight at 4°C with mixing.

2- 10µg of procaryotic recombinant rhMSF WT -aa was dialysed against 50mM sodium phosphate (NaH_2PO_4) + 150 mM NaCl pH 8.0. Using Pierce dialysed cassettes (cut-off 3.5kDa), overnight. The sample was then coupled to the activated resin then continued overnight at 4°C with shaking.

3- 10µg/2ml of Bovine Serum albumin (BSA) coupled to the activated resin then continued overnight at 4°C with shaking.

D-Antibodies used in this study for affinity column preparation**:**

1- Rabbit polyclonal identification antibodies (RpVSI) that recognise the MSF-unique sequence, RpVSI affinity column (0.5ml): affinity resin used; 1µg/ml RpVSI. Prepared in house

2- TYN 1.2. Mouse monoclonal antibody to MSF-aa, purified on protein L. prepared in house, 10µg/ml (Working concentration).

E- Washing buffer: 20mMTris – HCl pH 7.4

F- Blocking buffer: 50mM Tris buffer pH 10

G- Eluting buffer: 0.1 M Glycine pH 2.7, and 20mM Tris – HCL + NaCl 2M pH 7.4.

2.3.3.2 Methods:

Preparation of the RpVSI column is described here as an example, other columns were prepared by the same method.

2.3.3.2.1 RpVSI chromatography affinity column preparation:

The bottle of resin (Reactigel, Pierce) was equilibrated to room temperature before opening to ensure moisture did not condense on the product, decreasing its activity level for later use. 1ml of Reactigel resin (pierce CDI – Agarose) was used. It was then washed with ice cold water to remove acetone. The agarose was then suspended in a solution of the ligand e.g. MSF specific rabbit polyclonal antibody

(RpVSI) at 1µg/ml. The mixture was then transferred to a plastic bijoux bottle, sealed with parafilm and then placed in a 50ml tube and allowed to couple overnight at room temperature on rotator drive mixer. The resin was allowed to settle and unbound ligand was then removed. The uncoupled sites were blocked by resuspending the resin in 50mM TrisHCl buffer pH10 for several hours (3-4 hours) at room temperature. Finally the coupled agarose was washed with 3ml of PBS containing 0.05% (w/v) sodium azide and stored, sealed with parafilm at 4°C.

2.4.2.2.2 RpVSI affinity purification

Sample preparation: 5ml of TYS conditioned medium (CM) was dialysed using Pierce dialysis cassettes (cut-off 3.5kDa) against 20mM Tris HCl 7.4. 2 x 4 litres overnight at 4°C, the buffer was changed after the first hour.

The RpVSI column resin was then equilibrated with the Tris buffer, for 2 hours at 4ºC with mixing. The test proteins were then added to the resin; the tubes were then sealed with parafilm and mixed overnight at 4°C. The resin was then allowed to settle or was spun at 1,000 rpm for 1 minute and the unbound, soluble material removed. The resin was then washed for 1 hour at 4°C with the Tris buffer, allowed to settle and the wash buffer removed. The resin was then washed again with 4 x 1ml of Tris buffer, the resin was pelleted between each wash by a 1 minute spin at 1,000rpm. The resin was then eluted with 5 x 1ml of 0.1 M Glycine pH 2.7 the eluted material was pipetted into tube containing 50 µl 1 M Tris-HCl pH 8.7. The column was re-eluted with an increasing salt gradient formed by mixing 20mm Tris HCl pH 7.4 with 20mm Tris HCl pH 7. The affinity resin was washed until the pH was neutral and then stored in PBS containing 0.05% (w/v) sodium azide at 4°C. Eluted fractions were tested for MSF bioactivity in the transmembrane migration (Boyden chamber) assay.

The same method was used to prepare the other affinity columns with different ligands which have been used in this study.

2.4 Immunohisochemistry Study

2.4.1 Detection of MSF by immunohistochemistry (IHC)

Immunohistochemistry is the localisation of antigens in tissue sections by the use of labelled antibody as specific reagents through antigen-antibody interactions that are visualised by a marker such as a fluorescent dye, enzyme, radioactive element or colloidal gold. Immunohistochemistry for MSF was carried out using a protocol developed by Cell and Molecular Biology (Dr Ana Schor), Dental School, Dundee University.

2.4.1.1 Materials

2.4.1.1.1 Slide staining:

- Xylene – BDH AnalaR; VWR International, Leics., Cat. no. 102936H (2.5l).
- Ethanol – BDH AnalaR; VWR International, Cat. no. 101077Y (2.5l).
- PBS – PBS tablets; Sigma-Aldrich, Dorset, Cat. no. P-4417.
- Tween 20 – Sigma-Aldrich, Cat.no. P1379.
- Hydrogen Peroxide 30% v/v; Cat.no: 103665H (1l). VWR Ltd, Leicestershire.Uk
- ImmunoPen – DakoCytomation Pen; DakoCytomation Ltd., Cambs, Cat.no. S2002.
- Normal Goat Serum – Vector Labs. Peterborough, Cat. no. S1000.
- Avidin/Biotin Blocking Kit – Vector Labs, Peterborough, Cat. no. SP-2001.
- Normal mouse IgG (as negative control). DAKO, X0931 (100mg/L).
- ABC Kit – Vectastain ABC kit, Standard Elite. Vector Labs., Cat no PK6100.

- DAB – (3, 3-diamino benzidine) Sigma-Aldrich, Cat no. D-5637.
  10% v/v or w/v solution made up in PBS heated to approx 50ºC. Aliquoted into 800µl aliquots in Cryotubes.(NUNC, Cat. No. 363401), quick-frozen at -80ºC for 1 hour then stored at -20ºC.

- Haematoxylin – Haemalum, Mayer. Triangle Biomedical Sciences (TBS), Lancashire, Cat.no. PS50/c.

- Blueing agent – Thermo Shandon, Cheshire, Cat. no. 6769001.

- DPX – BDH; VWR International, Cat. no.360292F (100ml).

2.4.1.1.2 Antibodies and Negative Controls:

The following antibodies and controls were used:

Primary antibodies:

- Mouse monoclonal antibody HYB7.1 batch 1. 40µg/ml. (Hybridoma antibody to total MSFWT) (stock prepared in house, 0.243mg/ml).

- TYN 1.2 (batch 3) 40µg/ml. Mouse monoclonal antibody to MSF aa-, purified on protein L. prepared in house (Stock conc. 221µg/ml).

- Normal mouse IgG (nMIgG); negative control Cat no: X093101, Lot no: 0033117, (Dako, Ely, Cambridgeshire, UK). Stock Conc 100µg/ml, (40µg/ml in 20% (v/v) NGS)

Secondary antibodies:

- Biotinylated goat anti-mouse IgG. Code BA-9200, lot.T0206. Stock Conc1.5 mg/ml, (Vector Labs Ltd, Peterborough, UK). (1:5 diluted in 20% (v/v) N.G.S).

2.4.1.2 Methods

2.4.1.2.1 Immunohistochemistry:

- The sections were dewaxed in xylene for 5 minutes.
- The sections were then rehydrated through 100% -95% - 70% v/v ethanol for 2 minutes, in each solution.
- The sections were incubated with 3% hydrogen peroxide (H_2O_2) in PBS for 20 minutes to inhibit endogeneous peroxidise activity. The slides were then rinsed twice in PBS, 5 minutes per rinse.
- The sections were then outlined with Immuno pen.
- The sections were incubated in 20% v/v normal goat serum (NGS) diluted in PBS for 30 minutes.
- Sections were then rinsed with PBS using a wash bottle.
- The slides were then rinsed twice in PBS, 5 minutes per rinse.
- The sections were then incubated in avidin block from the avidin-biotin blocking kit for 15 minutes.
- Sections were then rinsed with PBS using a wash bottle, and then rinsed twice in PBS, 5 minutes per rinse.
- Sections were then incubated in biotin block for 15 minutes.
- The biotin solution was then gently removed from each section..
- The sections were then incubated overnight at 4ºC in the primary antibody, monoclonal 7.1b1 40µg/ml, diluted in 20% v/v NGS in PBS.

The slides were then **e**quilibrated at room temperature for 1 hour.

- The primary antibody was then removed from the sections by rinsing gently with PBS using a wash bottle.
- Rinsed the sections in PBS-T (PBS + 0.05% v/v Tween 20) for 5 minutes twice.
- Then rinsed the sections in PBS for 5 minutes.
- Incubated the sections in secondary antibody biotinylated anti-Mouse IgG at 6µl/ml in 20% v/v NGS in PBS for 40 minute.
- Prepared Vectastain (ABC) complex during incubation at 12µl, 2 drops from reagent A (Avidin) and 2 drops from reagent B (Biotinylated) per 5 ml PBS for 30 minutes at 37ºC.

- The sections were then rinsed gently with PBS from a wash bottle.
- The sections were then rinsed twice in PBS-T (PBS + 0.05% v/v Tween 20) for 5 minutes.
- The sections were then rinsed in PBS for 5 minutes.
- The sections then incubated in ABC complex for 30 minute.
- The sections were rinsed gently with PBS from a wash bottle.
- The sections were then rinsed twice in PBST (PBS + 0.05% v/v Tween 20) for 5 minutes.
- The sections were then rinsed in PBS for 5 minutes.
- The slides were immersed in DAB (diaminobenzidin) made up of 400 ml PBS, 800 µl DAB, 400 µl of H_2O_2 for 10 minutes, with agitation.
- The sections were then washed in running tap water.
- The sections were then counter-stained with Mayer's Haematoxyline for 30 seconds.
- The slides were rinsed in running tap water until the water ran clear.
- The sections were incubated in blueing agent for I minute then running in tap water.
- Sections were then dehydrated through 95% (v/v), 100% v/v ethanol for 1 minute in each, then in 100% v/v ethanol for 2 minutes.
- The slides were soaked in xylene for 5 minutes, then cover slip with mounting DPX.

2.4.1.2.2 Quantification and Assessment of MSF expression in salivary gland Tumour and OSCC tissue sections.

Duplicate sections of each specimen were stained and assessed by 2-4 independent observers. The final results were obtained by consensus. MSF staining was first evaluated at x100 magnification, scanning the whole section. The overall distribution of staining was recorded (e.g. homogeneous/heterogeneous, tumour cell- and/or stromal cell-associated, etc) and the following semi-quantitative parameters were then evaluated by comparison to pre-selected calibration slides:

MSF overall grade (0-3): Specimens were initially graded as negative (grade 0), weak (grade 1), moderate (grade 2), or strong (grade 3) positive. At least 10% of the whole area stained was chosen as the cut-off point between grade 0 and grade 1. Although such overall grade evaluation includes epithelial and stromal compartments,

it reflects mainly the former, as higher magnification is required to assess the stroma. The epithelial and stromal compartments were then evaluated individually. The final results were obtained by consensus.

MSF % of area stained (0-100): MSF expression in epithelial and invasive tumour front (ITF) for OSCC was defined by four indices. The percentage of total area stained (1-100%) was estimated, and the intensity of the staining was graded from 0 (negative) to 3 (strong) by comparison to calibration slides. The highest (hot spot) intensity (present in at least 10% of the epithelium) was also recorded. Final score (0-300) was derived by multiplying the % area stained by the intensity of the staining. Heterogeneous staining was common and this is reflected in the final score; for example, 50% area stained with intensity 2 and 20% area with intensity between 2 and 3 (2.5) gives a final score of 150.

Stromal MSF expression was classified, using higher magnification, as either positive or negative for three constituent stromal cell types: fibroblasts, microvascular and inflammatory cells.

MSF expression in NSG. The staining of the various cell types comprising the epithelial compartment of histologically normal salivary glands (NSG) adjacent to tumours was similarly classified as either positive or negative.

MSF expression in Hp. The staining of epithelial hyperplasia was similarly classified as MSF overall grade (0-3**)** for both basal and suprabasal cell layers.

The protein MSF is visualised as a brown colour due to the use of 3, 3'diaminobenzidine (DAB solution).

2.4.2 Immunocytochemistry (ICC) of Cultured Cell Lines:

Cell lines used for immunocytochemical staining were prepared by two methods:

- Plated onto a plastic surface, namely chamber slides and 24 well plates (Nunclon).
- Plated within and/or on a 3D collage type I matrix (cell pellets).

Cells were plated onto the plastic surface at densities of 5-9 x 10^4cells/cm^2 and incubated for 1-3 days under standard conditions, until reaching approximately 70-90% confluence in 1-3 days, as convenient for the staining. The medium was then removed, cells were washed twice with PBS or Hanks and then fixed with 2% (v/v) formalin in PBS for 30 minutes at room temperature followed by permeabilisation with 100% (v/v) methanol for 10 minutes at -20°C (methanol stored at -20°C).

After washing twice with PBS, endogenous peroxidase activity was blocked by immersion in 3% (v/v) hydrogen peroxide in PBS for 20 minutes and non-specific binding was blocked by incubating in 20% (v/v) normal serum in PBS (same species as secondary antibody) for 30 minutes.

Sections (or wells) were ringed using an immuno pen (Dako, Demark) to minimise the volume of reagent required. To reduce non-specific background, sections were pre-treated with an Avidin/Biotin blocking step (Avidin/Biotin Blocking kit, Vector Labs, Peterborough, UK). Following optimisation, the appropriate dilution of primary antibody was made using 20% (v/v) normal goat serum in PBS and incubated overnight in a humidified chamber at 4°C, followed by 1 hour at room temperature. Controls were incubated with normal serum IgG from the same species as the primary antibody was raised in. Secondary antibody incubation was for 1 hour at room temperature in a humidified chamber, the appropriate dilution was made in 20% (v/v) normal goat serum PBS. Staining was visualised using the avidin-biotin enzyme complex (Vectastain ABC Kit, standard Elite Vector Labs, Peterborough, UK).and incubation with DAB (3’3’- diaminobenzidine), a substrate for the enzyme (Sigma, Dorset,UK) Slides were counterstained with haematoxylin and Blueing agent before being cover slipped with mounting medium (Aqueous mount Sigma, Dorset, UK) for plastic and DPX.

2.5 Tissue Culture:

2.5.1 Materials:

2.5.1.1 Chemicals and reagents:

- Eagles Minimum essential Medium (MEM), lot no. M0275.
- L-glutamine, Lot no.G-7513 was purchased from Sigma-aldrich, UK.
- Donor Calf Serum (DCS), batch no. 0, lot no. APD21173 was from Thermo-Fisher Perbio, Hyclone, UK.
- Hank's solutions free of calcium and magnesium, (Lot no.H-4641), Sigma- Aldrich, UK)
- $NaHCO_3$ Lot no. 301515V, Merck. BDH, Germany.
- Trypsin solution was prepared from trypsin (lot no. T4549), Ethylene glycol tetraacetic acid (EGTA) (lot no. E-4378) and PBS tablet (lot no. P-4417). Sigma-Aldrich, Uk.
- Freeze mix was prepared by 15% (v/v) heat treated DCS and Dimethyl Sulfoxide (D-5879, Sigma- Aldrich, UK).
- Tissue culture plastic ware was purchased from SLS (Nottingham, UK).
- Haematoxyline was from Mayer's; distributed by Bios-Europe Lancs, UK, Cat no PS50C.

2.5.1.2 Cells:

The human salivary tumour cell line (HSG) and oral tumour cell line (TYS) which was derived from an oral squamous cell carcinoma of the floor of the mouth and were both a gift from Professor Mitsunobu Sato (Department of Maxillofacial Surgery, School of Dentistry, University of Tokushima School of Dentistry, Japan) (Shirasuna *et al.,* 1981). The human endothelial cell line (Endo 742) was a gift from MJ O'Hare Ludwig cancer Research Institute, London (O'Hare *et al*,. 2001). Human foreskin fibroblast (FSF44) cultures were established in our laboratory from health male donors and cultured as previously described (Schor, 1980).

Cells were maintained in cell culture using Eagle's Minimum Essential Medium (MEM) growth medium supplemented with 15% (v/v) Donor Calf Serum (DCS) and 1% (v/v) Glutamine, as previously described (Motegi *et al*,. 2008). Sub-culturing, cryopreservation and resuscitation of the frozen cell lines was performed as described below.

2.5.1.3 Collagen cell pellets.

Materials:

10x MEM (Gibco/Sigma), 7.5% $NaHCO_3$, Collagen 2.2mg/ml, Cells to be used (HSG, TYS), HBSS, Normal growth medium for cells to be used and trypsin (EGTA).

Method:

For each 2ml collagen cell pellet 2x 90mm dish of cells (1 dish for in gel and 1 dish on gel) were used (2 x 10^6 cell/dish).

For in gel: The normal growth medium was aspirated from the cells (1 dish). The cells were then washed twice with 3mls of HBSS (Hanks Balanced Salt Solution) and this was then aspirated from the dish. Then 2ml of 0.05% (w/v) trypsin in EGTA/PBS was added to the dish and the dish was then placed onto the hot-plate. After 5 minutes (or when the cells had rounded and floated up from the dish) the effect of the trypsin was reversed with normal growth medium. The floating cells were then pipetted into a universal and the cells pelleted in a centrifuge at 900rpm for 5 minutes. The medium was then aspirated and the cell pellet resuspended in 100µl of growth medium.

After the cells were resuspended, a 2ml collagen gel was prepared (200µl of 10x MEM, 100µl of 7.5% Na HCO_3, 1.7ml of 2.2mg/ml collagen), this was then added to the cells and allowed to set in the universal. After 30 minutes 10ml of normal growth medium was added to the collagen gel and the universal placed into the incubator for 3-4 days (the lid was loosened to allow gas exchange).

For on gel: On day 3-4 a further dish of cells was trypsinised (as above). After the cells were pelleted they were resuspended in 5mls of medium. The medium from the collagen pellet was removed and replaced with the 5mls of cell suspension. This was placed in the incubator and after 30 minutes a further 5mls of growth medium was added. After a further 24 hours the collagen gel was washed and fixed overnight at 4ºC with 10% (v/v) formalin buffered saline. The fixed pellets were sent to the pathology department, Ninewells hospital, Dundee for paraffin embedding.

2.5.2 Methods:

2.5.2.1 Cell culture

Cells were subcultured 2-3 times a week (1:3 or 1:4) and the medium was changed every second day. Confluent cultures were frozen for storage by the following protocols**:**

2.5.2.1.1 Subculturing

The cells on 90mm plates (Nunclon) were washed with Hanks balanced salt solution (HBBS) (4mls), trypsinised (EGTA) (2mls) at 37ºC for 5 minutes and neutralised with serum containing medium (5mls/plate). The cell suspension was spun at 900 rpm for 5 minutes. The pellet was resuspended in medium and split to other dishes and then incubated at 37 ºC and 5% CO_2.

2.5.2.1.2 Freezing down stock cultures

The confluent cells were washed with Hanks (4mls), trypsinised (EGTA) (2mls) at 37ºC for 5 minutes and neutralised with serum-containing medium (5mls/plate). The cell suspension was spun at 900 rpm for 5 minutes. The pellet was resuspended in freeze mix in a cryovial (Nunclon) and stored at -80 ºC.

2.5.2.2 Cell number determination:

Cell numbers (TYS, HSG, FSF44, and Endo 742) on plastic or gelatin coated dishes were obtained as follows: Cells were washed twice with 4ml Hanks balanced salt solution (HBBS). The cell layer was then trypsinised with 2ml trypsin solution (EGTA) and incubated at 37°C for 5 minutes. A single cell suspension was achieved by pipetting onto the monolayer. This suspension was aspirated from the dish and transferred to a 25ml universal containing 5ml of 15 % (v/v) DCS MEM. A 100µl aliquot of the cell suspension was then transferred to a Coulter vial containing 9.9mls of Isoton (Beckman Coulter. Germany). The total number of cells in 100µl of the cell suspension was determined using a Coulter particle counter.

2.5.2.2 Boyden chamber Cell migration Assay.

The migratory response of target cells to putative motogenic factors is most commonly studied *in vitro* with the transmembrane (Boyden chamber) assay. This experimental protocol may be used to distinguish between chemokinesis (random cell motility) and chemotaxis (directed cell motility toward increasing concentration of

soluble attractants) (Zigmond and Hirsch, 1973). The Boyden chamber assay is based on two chambers separated by a filter (a polyvinylproplene-free polycarbonate membrane) through which cells can migrate. The polycarbonate membrane used in the transmembrane assay does not support cell attachment and must consequently be coated with an adhesive compound, gelatin (denatured type I collagen) being a popular choice. Different concentration of chemo-attractants can be set up by placing in the lower chamber, so cells from the wells of upper compartment migrate into the lower compartment wells in which chemo-attractants are present through the pores of the membrane. Cell migration is quantified by simply counting migrated cells under a light microscope after staining.

The benefit of the Boyden chamber benefit is that it discriminates between chemo-kinetic and chemotactic influences. In addition, the Boyden chamber assay is somewhat time saving in that cells migrate through a porous membrane within a few hours (4-6 h) which is considered a short time in comparison with the time required for cells to proceed through a cell cycle, furthermore the Boyden chamber assay allows for cell motility analysis on a basis without consideration of the effect of cell proliferation on their results. Some of the important factors of Boyden chamber assay are considered to be the number of cells to be loaded on the chamber, the type and concentration of the attractant, the pore size of the membrane and the incubation time. A number of different Boyden chamber devices are available which vary in their sample size and quantitation method.

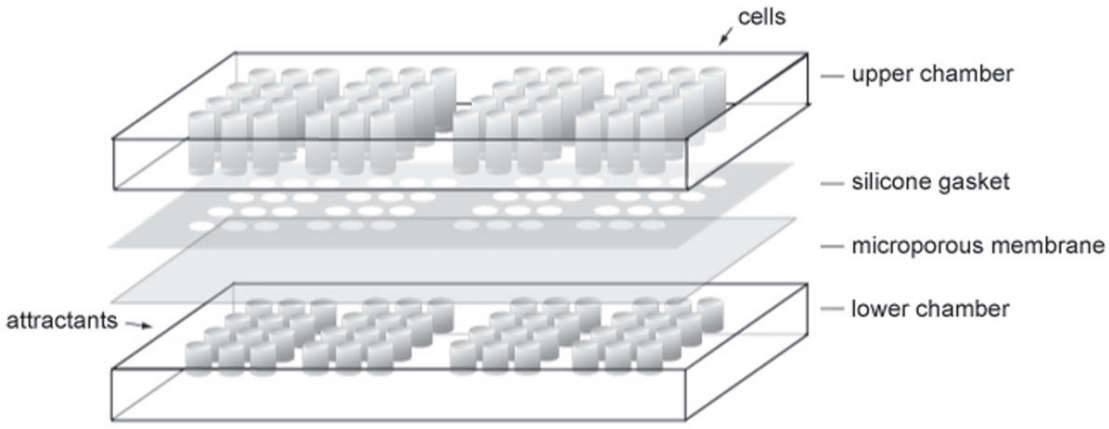


Figure 2.1: Neuro Probe standard 48-well chemotaxis chamber (adapted from Chen, 2004)

Chamber Construction:

To construct the assay a set volume (30µl) of MEM containing 2µg/ml bovine serum albumin (BSA) ± a known concentration of the factor under investigation (forming a small positive meniscus) was added to the bottom wells of the 48-well chamber (Neuro Probe, Inc., Gaithersburg, MD, USA) (Fig 2.1). A polyvinylpropylene free polycarbonate Nucleopore membrane (0.5-0.8µm) (Whatman Ltd, Maidstone, Kent, UK) coated with native type I collagen was placed, shiny side down, over the filled wells. The chamber gasket and upper well section were fixed firmly with screws. The apparatus was equilibrated for 30-45 minutes at 37°C in a humid atmosphere of 5% CO_2.

Cell preparation:

Stock dishes of confluent cells were washed twice in HBSS. Cell were trypsinised with 2ml trypsin solution (EGTA), the trypsin was then inactivated with growth media containing 15% (v/v) DCS and cells pelleted by centrifugation at 900rpm for 5 minutes. Cells were then resuspended in serum-free MEM containing 2µg/ml BSA, cell numbers were counted using the Coulter Counter and resuspended at 5 x 10^5 cell/ml. 50µl of this cell suspension was added to each of the upper wells. The chamber was then incubated at 37°C in humid atmosphere of 5% CO_2 for 5 hours.

Membrane Fixation and staining:

The chamber was disassembled and the membrane removed using forceps. The membrane was gently rinsed in PBS and fixed in methanol for 10-15 minutes. Cells were stained overnight with Mayers Haematoxylin. Membranes were washed repeatedly in tap water and then cut into 4 sections. The unmigrated cells on the upper surface of each membrane section were removed by wiping off onto damp Whatman No.1 filter paper. Membrane sections were mounted onto glass slides under cover slips using Glycergel aqueous mountant (Sigma, Bucks**,** UK).

Measurement of migration:

Migrated cells were counted microscopically (magnification x200) under bright field illumination. Six replicate wells were used per variable, with the number

of cells counted in 3 random fields per well (i.e. total of 18 fields) used to calculate mean cell number per field ± standard deviation

Methodology:

Migration assays were performed using a modified Boyden chamber assay as previously described [Schor et al, 1996; 2006]. The bottom wells of the 48-well chamber (Neuro Probe, Inc., Gaithersburg, MD, USA) were loaded with 30 µl of different concentrations (1pg/ml, 10pg/ml, 100pg/ml, 1ng/ml, 10ng/ml, and 100ng/ml) of rhMSF dissolved in SF-MEM with 2µg/ml bovine serum albumin (BSA). The 0.8 µm pore polycarbonate Nucleopore membranes (Whatman Ltd, Maidstone, Kent, UK) (coated with native type I collagen) were then applied and the upper wells of the assembled chamber loaded with 50µl of HSG cells (at 1.2 x 10^6 cells/ml) in SF-MEM containing 2µg/ml BSA. After a 5 hour incubation period at 37°C in a humidified CO_2 incubator, the membranes were removed, fixed in methanol and stained with Mayer's Haemotoxylin. The cells remaining on upper surface of the membrane were scraped off and the cells that had migrated through the pores to the under surface were assessed microscopically (x200) under bright field illumination. Six replicate wells were used per variable, with the number of cells counted in 3 random fields per well (i.e. total of 18 fields) used to calculate mean cell number per field ± standard deviation.

2.6 Isolation of proteins produced by cultured cells

2.6.1 Conditioned Medium Collection and Storage:

Cells (TYS, HSG) were grown to confluence on 90mm dishes**.** Confluent cultures were rinsed once with 4ml of Hanks and then twice with 5ml serum-free MEM. Dishes were incubated for 2 hours at 37°C in 5 ml of SF-MEM. The medium was discarded and dishes were washed again with SF-MEM. Cells were then incubated in fresh SF-MEM for 48 hours. The CM was collected, spun for 5 minutes at 900rpm and the supernatant was collected and stored at -20°C.

2.6.2 Cell lysate preparation

TYS cells plated on five 90mm dishes (90% confluences) were washed with cold PBS, the cells were lysed with 500 µl/plate of an RIPA buffer (50 mM Tris–HCl

pH 7.4, 150 mM sodium chloride, 1% (v/v) Triton X-100, 0.1 % (w/v) SDS, 1% sodium deoxycholate, 5 mM EDTA). Cells were incubated on ice until completely lysed, then surface of the Petri-dish was then scraped with 1mL syringe stopper. The lysates were spun in a bench-top centrifuge at full speed for 10 min, and the resultant pellet was discarded. The protein extract was then added to the resin following the same methods as for RpVSI immunoprecipitation as described in page (66). The eluted fractions were tested to identify MSF by using Indirect ELISA and Western blot. Detect protein by ELISA or Western or place in –20 freezer.

2.6.3 Cell Membrane Protein Extraction:

2.6.3.1 Materials

Mammalian cell pellet, protease inhibitor (Roche Complete Mini, EDTA-free. lot; 11051600), Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce # 89826). Target cells as in (Table 2.2).

Table 2.2: Cell types

Tumour cell lines	Definition
FSF44	Human foreskin fibroblast cells
HSG	Human salivary gland tumour cells
TYS	Human oral tumour cell line
Endo 742	Human Endothelial cells

2.6.3.2 Methodology

Membrane proteins were extracted from pellets of cultured cells using the Mem- PER Eukaryotic Membrane Protein Extraction Reagent Kit from Pierce, essentially according to the manufacturer's instructions. Cells were cultured as previously described (page 76) and collected as cell pellets and frozen at -70ºC. 5 x 10^6 cells for each cell line were extracted. The mixture was then centrifuged at 10,000g and incubated at 37°C to isolate the hydrophobic proteins (bottom layer) from the hydrophilic proteins (top layer). The hydrophobic fraction contained the majority of membrane proteins required for downstream analysis. Identification of

integrin αvβ3 from membrane proteins extracted from these cell lines was by ELISA, Western blotting and Mass Spectroscopy.

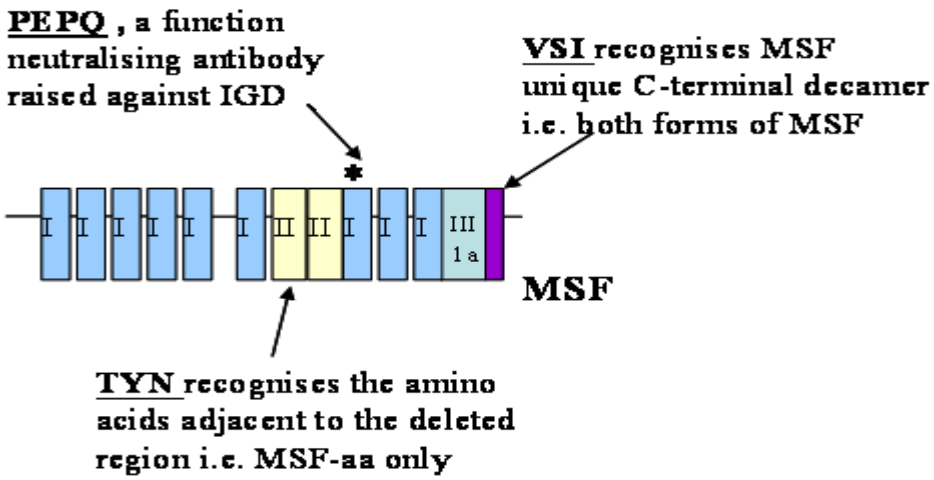
2.7 Statistical analysis:

Statistical analyses were carried out with the Prism 5 (Graphpad Inc. La Jolla, CA, USA) software package. Each experiment was repeated a minimum of two times. Differences among groups of tissues were determined either by chi-squared and Fisher's exact tests or one ANOVA Bonferroni Test and two-tailed Mann–Whitney tests, as appropriate. Differences in cell migration were analysed by ANOVA and Bonferroni tests. Significance differences were defined at 95% level of confidence ($P < 0.05$). Survival analysis was undertaken using Kaplan-Meier curve followed by the long rank test.

Table 2.3: Total primary and secondary antibody used in this study

Primary -Ab	Stock	origin	Secondary-Ab	origin
RpVSI	20µg/ml	In house	Rabbit anti-mouse HRP	DAKO
Goat Anti- human integrin αV lCD 51 AF1219	200µg/ml	R&D Systems	Rabbit anti-goat HRP	R&D Systems
Mouse Anti- human integrin β3 (CD61) mab	100µg/ml	R&D Systems	Rabbit anti-mouse HRP	R&D Systems
Anti-cell binding domain of Fibronectin antibody MAB1937	1mg/ml	CHEMICON	Goat anti-rabbit (GAR-HRP)	DAKO or Pierce
Anti-human Lipocalin (Goat Polyclonal AF1757)	200µg/ml	R&D systems	Rabbit anti-mouse RAM-HRP	DAKO
αvβ3 antibody MAB3050	500µg/ml	R&D Systems	Rabbit anti-goat RAG-HRP	DAKO
IGFBP7 antibody AF1334	100µg/ml	R&D Systems	Goat -anti- mouse ab	MSD Sulph-Tag
Mouse monoclonal antibody TYN 1.2	221µg/ml 288µg/ml	In house	Goat anti-mouse IgG	MSD Sulph-Tag
RpabVSI	1.28mg/ml	In house	Goat -anti- rabbit ab	MSD Sulph-Tag
MAB 2.1VSI	592µg/ml	In house	Biotinylated rabbit anti-mouse IgG	Dako
Mouse monoclonal antibody HYB7.1b1	592µg/ml 0.243mg/ml	In house	Biotinylated goat anti-mouse IgG	Dako
Normal mouse IgG	100µg/ml	Dako	Biotinylated goat anti-mouse IgG	Vector Labs
Mab PEPQ 1.1	61.4µg/ml	In house		

MSF Antibodies:



3. Chapter three: Expression and bioactivities of MSF in vitro

3.1 Introduction:

Recombinant MSF exhibits a number of potent bioactivities in vitro and in vivo relevant to cancer progression and wound healing. This study focusses mainly on the motogenic activity of MSF, studying the stimulation of migration/invasion by tumour cells, fibroblast and endothelial cells. Schor *et al,* (2003) indicated that mutations of IGD to DGI in fibronectin type I module at 7 and 9 (7FnI and 9FnI) eliminate the motogenic activity of MSF on fibroblasts migration. Therefore, the motogenic activity of MSF on fibroblasts is mediated by two IGD amino acid motifs located in MSF modules 7FnI and 9FnI. Ellis *et al,* (2010) reported that MSF contain a cryptic motogenic activity in the 3FnI and 5FnI modules released when MSF is degraded into small fragments.

Another active motif has been reported in MSF. This motif is a putative zinc binding amino acid motif (HEEGH), located in module 8FnI of MSF and fibronectin, and is essential for fibronectin proteinase activity (Houard *et al.,* 2005). Houard *et al,* (2005) reported that this motif is also necessary to stimulate the migration of a breast tumour cell line (MCF-7), as the proteinase and motogenic activities of MSF were abolished by the mutagenesis of the two histidine residues to phenylalanine (FEEGF).

3.2 Aims:

The objectives of this study were to:

1- Determine the effect of MSF on different types of cells (oral tumour cell line TYS, salivary gland tumour HSG, human endothelial cell Endo 742 and human fibroblast FSF44) in terms of migration.
2- Determine the specific amino acid motifs of MSF (IGD and/or HEEGH that are responsible for its bioactivity.
3- Determine the modulation of MSF bioactivity by antibodies (PEPQ and, TYN) and other proteins (NGAL, BP-7)
4- Determine the expression of MSF by oral tumour cell lines.

3.3 Materials and Methods:

3.3.1 Cells: The following human cells were used:

TYS: Oral tumour cell line (Shirasuna *et al.,* 1981)

HSG: Salivary gland tumour cell line (Shirasuna *et al.,* 1981)

Endo 742: Microvascular endothelial cells (O'Hare *et al.,* 2001)

FSF44: Foreskin fibroblasts (Schor, 1980)

Tumour cells HSG and TYS and endothelial cells Endo 742 were gifted from the laboratories where originated by Professor M. Sato and Dr Michael O'Hare, respectively. FSF44 fibroblasts were established in the laboratory of Prof S.L.Schor by explant culture from foreskin obtained from a healthy 1 year old male donor.

3.3.2 Proteins and antibodies

The proteins and antibodies used in this study are listed in Tables 3.1, 2 and 3;

Table 3.1: MSF and its mutant proteins

PROTEIN NAME	MUTATIONS	ABBREVIATED NAME
rhMSF+aa WT	NONE	rhMSF+aa
rhMSF-aa WT	15 amino acid deletion	rhMSF-aa
rhMSF+aa DGI 3,5	IGD 3 → DGI IGD 5 → DGI	MSF 3,5m
rhMSF+aa DGI 7,9	IGD 7 → DGI IGD 9 → DGI	MSF 7,9m
rhMSF+aa DGI 3,5,7,9	IGD 3 → DGI IGD 5 → DGI IGD 7 → DGI IGD 9 → DGI	MSF Qm
rhMSF+aa FEEGF	HEEGH → FEEGF	MSF Hm
rhMSF+aa DGI 3,5,7,9 FEEGF	HEEGH → FEEGF IGD 3 → DGI IGD 5 → DGI IGD 7 → DGI IGD 9 → DGI	MSF QmHm
rhMSF+aa DGI 7,9 FEEGF	HEEGH → FEEGF IGD 7 → DGI IGD 9 → DGI	MSF 7,9m Hm

Table 3.2: Proteins used in this study

PROTEIN NAME	ABBREVIATED NAME
Migration stimulating factor	rhMSF+aa rhMSF-aa
Neutrophil gelatinase-associated lipocalin	rhNGAL
Recombinant Insulin-like Growth Factor Binding Protein-7	rhBP-7
Insulin-like Growth Factor Binding Protein-7 purified from endothelial cell CM	eBP-7

Table 3.3: Antibodies used in the migration assay study

ANTIBODY NAME	ABBREVIATED NAME
MSF Function-neutralising antibody	PEPQ
Identification Anti total MSF antibody	VSI
Anti MSF-aa antibody	TYN

3.3.3 Methods:

- Cell culture
- Collection of conditioned medium
- Immunocytochemisrty
- Transmembrane migration assay (Boyden chamber).
- Affinity purification of Conditioned medium.
- Preparation of cell lysates

These methods are described in chapter two (Materials and Methods).

3.3.4 Statistical analysis:

Statistical analyses were carried out with the Prism 5 software package (Graphpad Inc. La Jolla, CA, USA). The distribution (parametric / non-parametric) of the data was determined with the Kilmogorov-Smiroff test. Since the data followed a normal distribution, differences among experimental groups were analysed by ANOVA and Bonferroni tests. Significance differences were defined at the 95% level of confidence ($P < 0.05$).

3.4 Results:

3.4.1 Effects of MSF on cell migration

The effect of MSF proteins was investigated in the transmembrane assay as described in Materials and Methods. Briefly, the proteins were diluted in serum-free-MEM containing 2µg/ml BSA (SF-BSA). SF-BSA was used as control (baseline) and the number of cells that had migrated was determined microscopically after a 5 hours incubation period. Experiments were repeated 3-6 times.

3.4.1.1 Effects of rhMSF+aa on cell migration

The motogenic effect of rhMSF+aa was tested on TYS, HSG and Endo 742 cells. Results are presented in (Fig. 3.1A, B, and C). By comparison, results previously published with fibroblasts (Schor et al., 2003) are shown in (Fig 3.1D). Different concentrations of rhMSF +aa had a significant stimulatory effect on the migration of all four cell types (Bonferroni's test). Significant stimulation was detected at concentrations of 0.1- 10pg/ml (Fig 1). In the case of TYS, Endo 742 and FSF44 higher concentrations induced less stimulation, reaching baseline levels at 500ng/ml -1µg/ml. Therefore, MSF biological activity displayed a bell shaped dose-response curve, with maximal stimulation of migration achieved at a concentration 100pg/ml. However, in the case of HSG cells all concentrations of MSF tested (1pg/ml-1µg/ml) exhibited significant motogenic activity by comparison to the negative control or baseline, with a plateau reached at 10ng/ml.

3.4.1.2 Effects of rhMSF-aa on cells migration

The effect of rhMSF-aa on the same four cell lines was investigated in the transmembrane migration assay as described above. Fig 3.2 shows that significant differences on the migration of TYS, HSG, Endo 742 and FSF44 occurred between baseline control levels and different concentrations of rhMSF-aa tested ($P < 0.05$; ANOVA and Bonferroni tests). These results are similar to those obtained with rhMSF+aa regarding dose-response patterns.

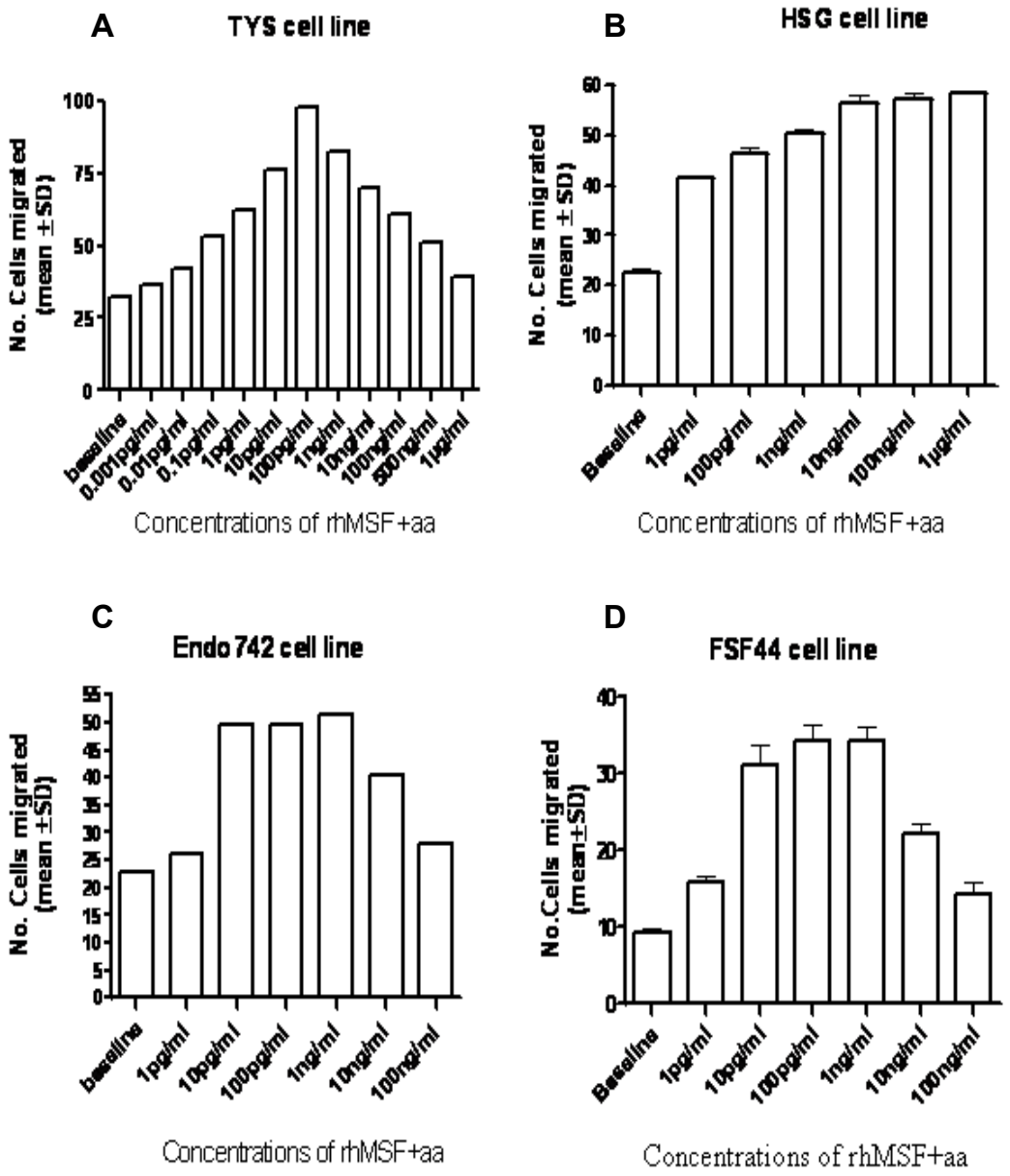


Figure 3.1: Effects of different concentrations of rhMSF+aa on the migration of TYS, HSG, Endo 742 and FSF44 cell lines.

Cells were plated on collagen coated filters and the number of cells that had migrated was determined after 5 hours. The minimal concentration of rhMSF+aa needed to stimulate migration was around 0.1 pg/ml for TYS, 1pg/ml for HSG (this was the lowest concentration tested), and 10 pg/ml for both Endo742 and FSF44. MSF biological activity on TYS, Endo742 and FSF44 displayed a bell shaped dose-response curve, with maximal stimulation of migration achieved at a concentration of 100pg/ml. However, in the case of HSG cells all the concentrations of MSF tested (1pg/ml-1µg/ml) exhibited significant motogenic activity by comparison to the negative control, with a plateau reached at 10ng/ml.

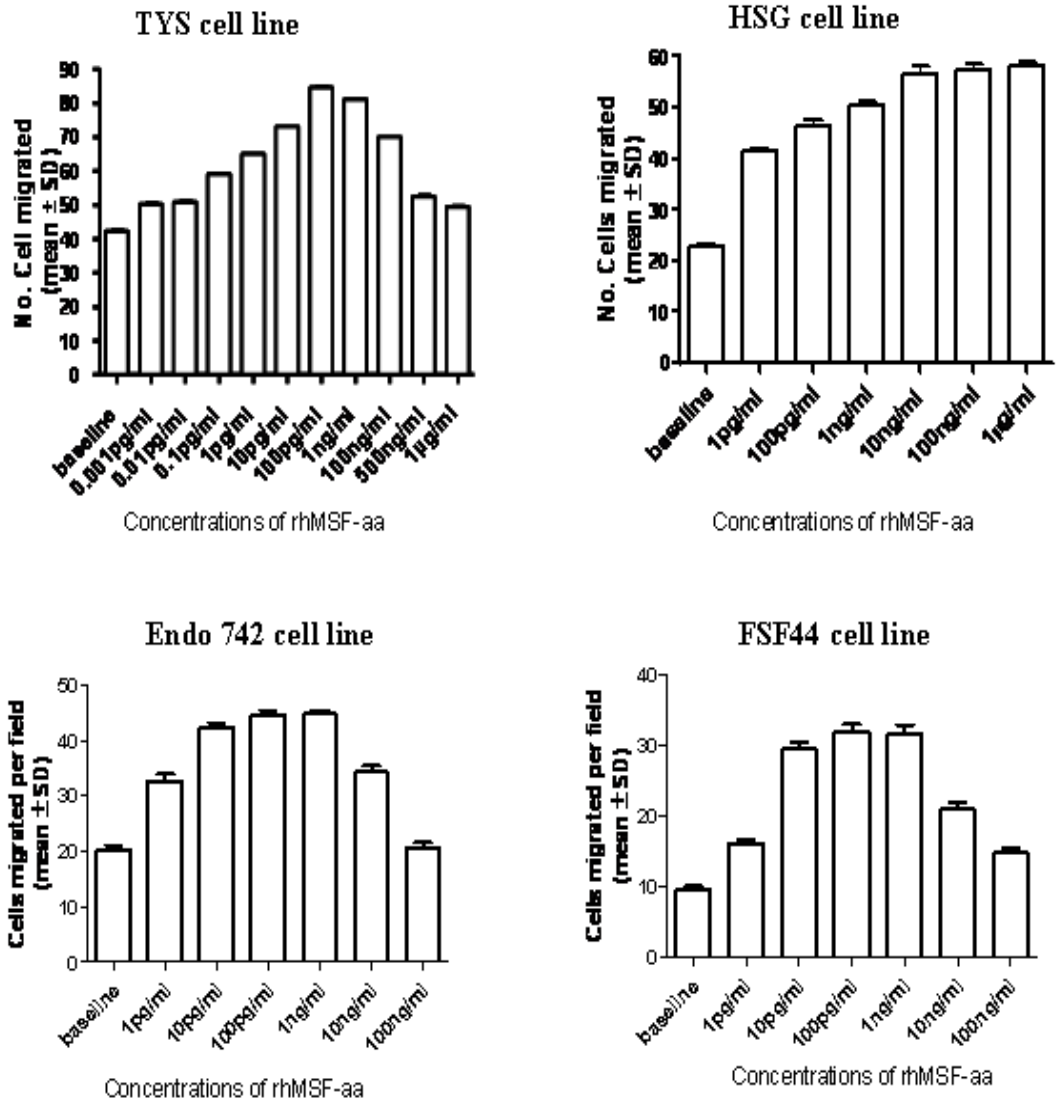


Figure 3.2: Effects of different concentrations of rhMSF-aa on the migration of TYS, HSG, Endo 742 and FSF44 cell lines.

Cells were plated on collagen coated filters and the number of cells that had migrated was determined after 5 hours. The minimal concentration of rhMSF-aa needed to stimulate migration was around 0.1 pg/ml for TYS, 1pg/ml (lowest concentration tested) for both HSG and Endo742, and 10pg/ml for FSF44. MSF biological activity on TYS, Endo742 and FSF44 displayed a bell shaped dose-response curve, with maximal stimulation of migration achieved at a concentration of 100pg/ml. However, in the case of HSG cells all the concentrations of MSF tested (1pg/ml-1µg/ml) exhibited significant motogenic activity by comparison to the negative control, with a plateau reached at 10ng/ml.

3.4.2. Bioactive motifs of MSF

rhMSF mutant proteins (Table 3.1) were produced by *in vitro* mutagenesis and prokaryotic expression (Schor *et al.,* 2003) and were made available for my study by SL Schor, AM Schor and SJ Jones, at the Cell and Molecular Biology Unit, Dundee Dental Hospital.

3.4.2.1 Effects of rhMSF mutants on cell migration

The biological activity of rhMSF mutants was compared in the transmembrane migration assay. The statistical analysis of the results for rhMSF mutants (including MSF 3,5m, MSF 7,9m, MSF 7,9m Hm, MSF Hm, of MSF Qm, and MSF QmHm) were similar in all experiments, proving there was no significant stimulatory effect of rhMSF mutants on the migration of TYS(Fig 3.3 & 3.4) and HSG cell lines (Fig 3.5A) (ANOVA and Bonferroni's $p>0.05$). Therefore, the biological activity of MSF appears to be mediated by both IGD and HEEGH motifs for the stimulation of both oral tumour cells (TYS) and salivary gland tumour cells (HSG). In contrast, data presented in (Fig 3.5B) indicates that the migration of Endo 742 was stimulated by rhMSF mutants involving either IGD or HEEGH motifs (MSF 3,5m MSF+aa 7,9m, MSF 7,9mHm, MSF Hm, and MSF Qm), whilst mutation of both the HEEGH and all four IGD motifs in MSF abolished the motogenic response of endothelial cells.

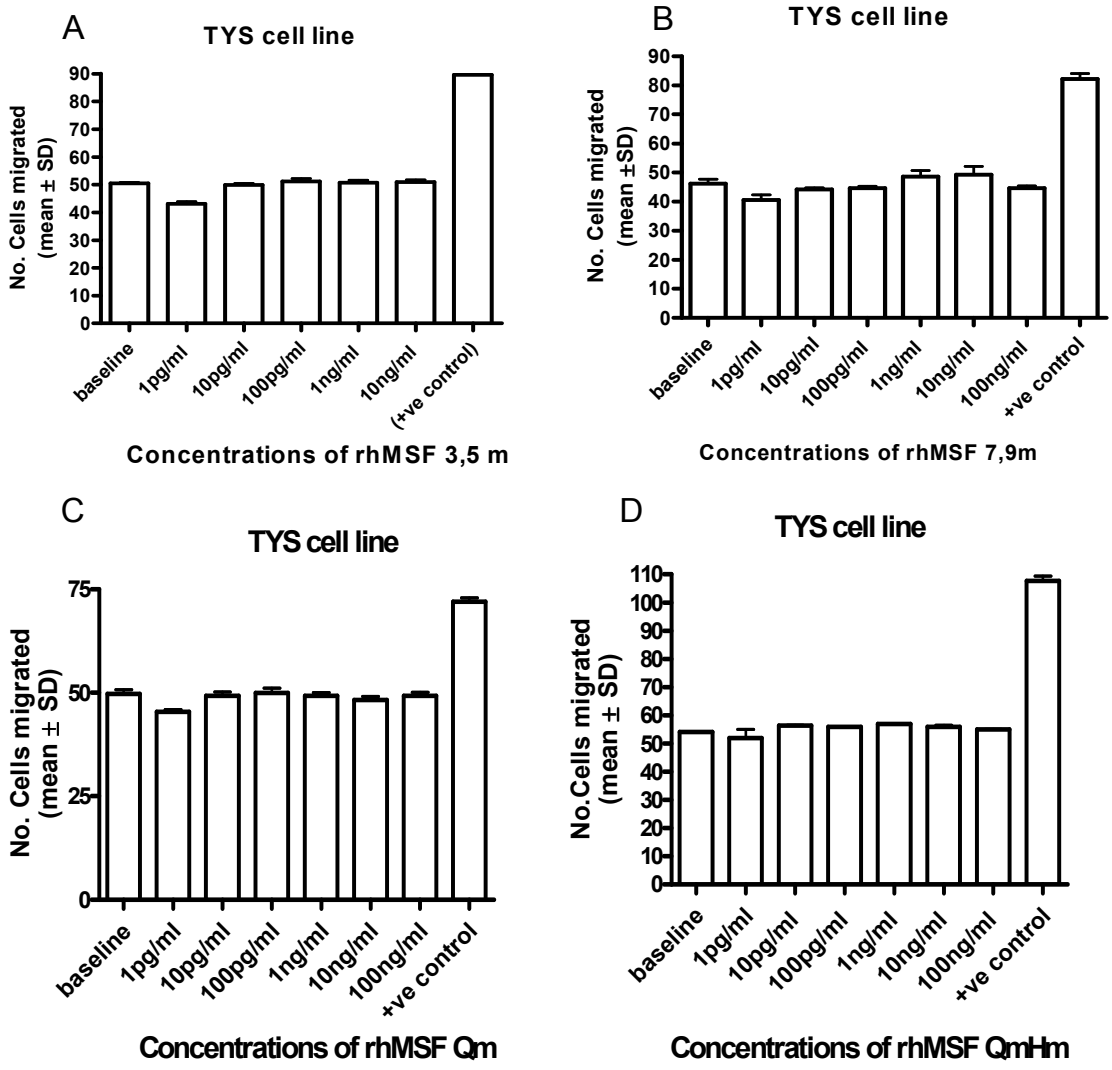


Figure 3.3: Effects of different concentrations of rhMSF mutants (3,5m, 7,9m, Qm and Hm) on the migration of TYS cell line.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. There was no significant effect of all these rhMSF mutants on cell migration by comparison to the negative control (Bonferroni's test)

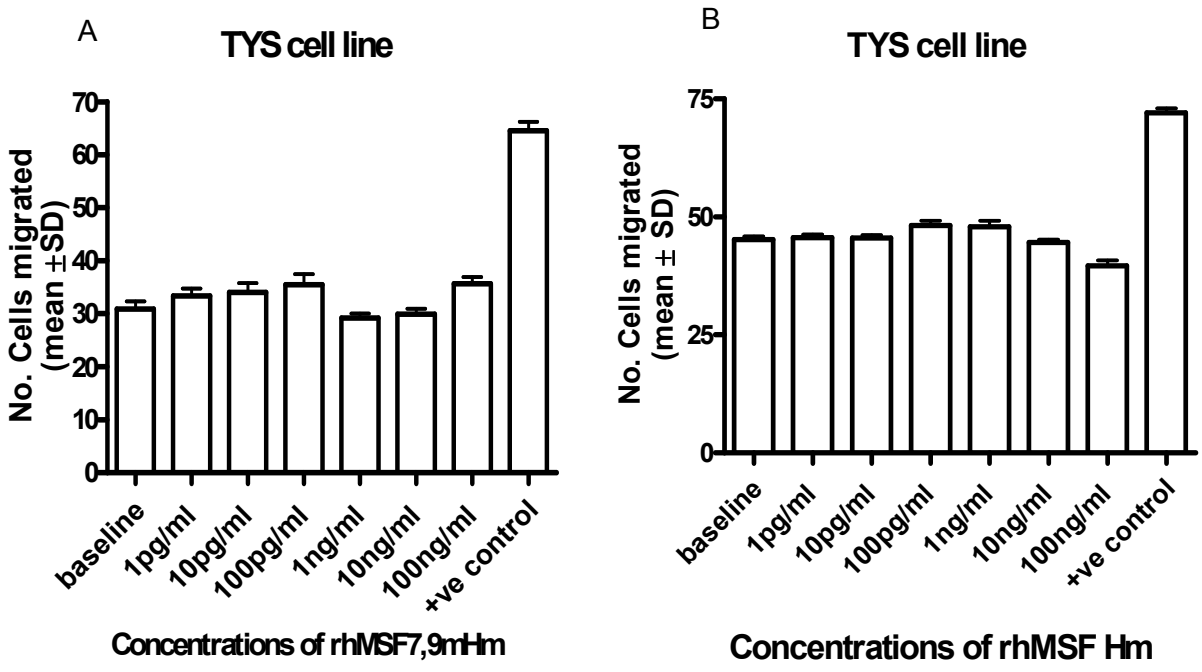


Figure 3.4: Effects of different concentrations of rhMSF mutants (7,9mHm and Hm) on the migration of TYS cell line.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. There was no significant effect of both mutants on cell migration by comparison to the negative control (Bonferroni's $P>0.05$).

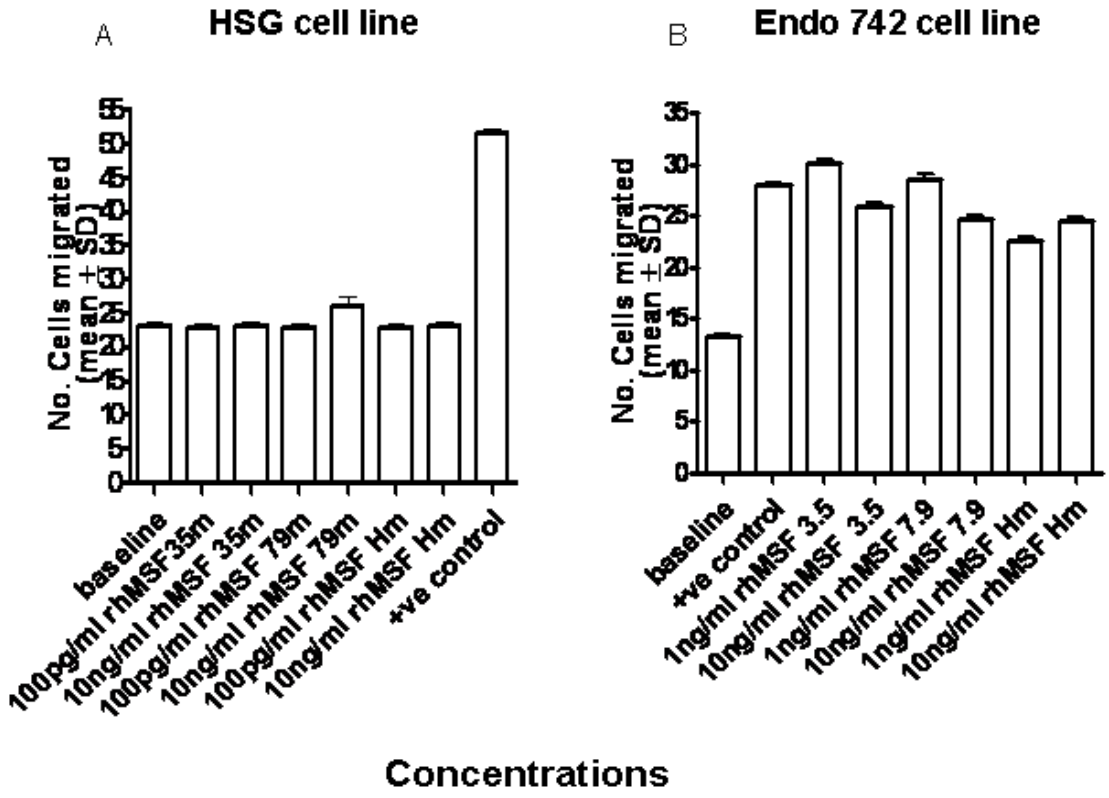


Figure 3.5: Effects of different concentrations of rhMSF 3,5m, 7,9m and Hm on the migration of HSG and Endo 742 cell lines.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. (A) There was no significant effect of all mutants on the migration of HSG cells by comparison to the negative control (Bonferroni's >0.05.). (B) There was significant effects of all mutants on the migration of Endo742 by comparison to the negative control (Bonferroni's $P<0.05$).

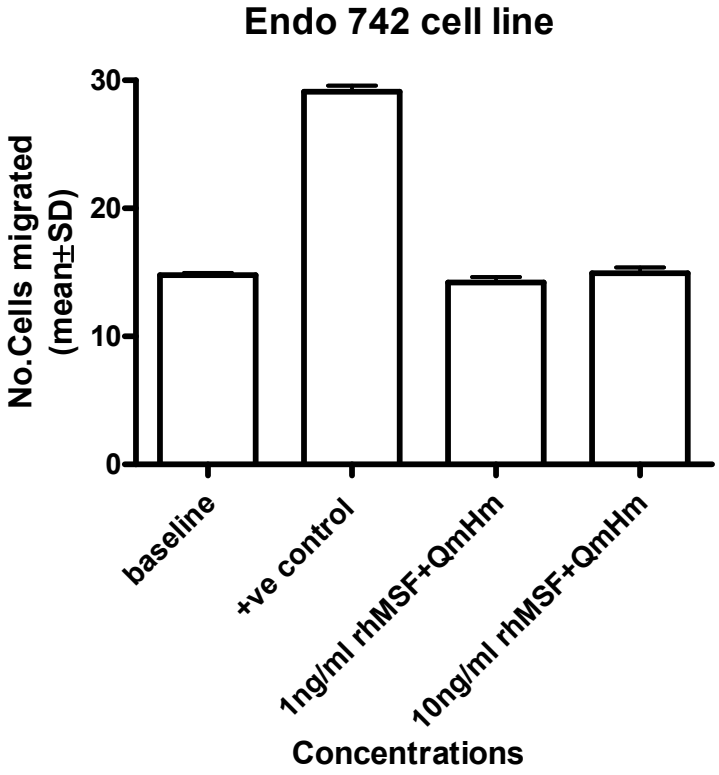


Figure 3.6: Effects of different concentrations of rhMSF QmHm on the migration of Endo 742 cell line.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. (A) There was no significant effect of rhMSF QmHm at (1ng/ml and 10ng/ml) on the migration in comparison to the negative control (Bonferroni's >0.05).

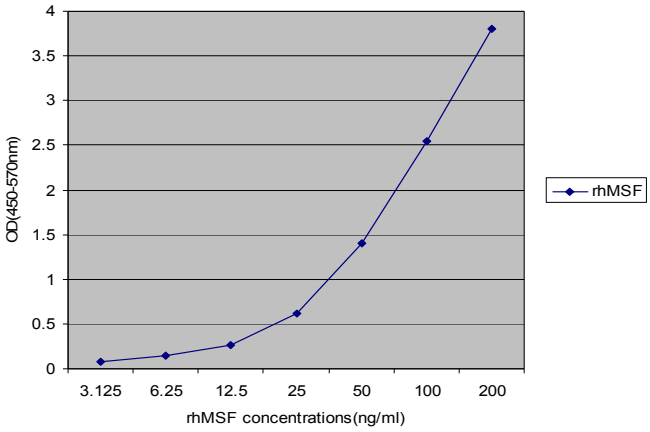
3.4.3 Characterisation of antibodies to MSF

Antibodies used for MSF identification (VSI, TYN) and function-neutralising (PEPQ) were produced by Schor and Schor (Principal Investigators) contracted to Borek Vojtesek (Schor *et al.,* 2003 and unpublished data). Characterisation of these antibodies was performed by Dr. B.Vojtesek, Dr. K. Kankova, members of the Cell and Molecular Biology Unit, Dental School (Dr. S. Jones, Dr. I. Ellis, Mrs. J. Cox, and Mrs. M. Florence. L Aljorani) and members of the Cancer Research Technology, Development Laboratory, London (Dr. D. Snary, Dr S. Foo) in collaboration with Schor and Schor or under their supervision. Results presented below are either published (Schor *et al.,* 2003) or with acknowledgment to the researchers involved, as indicated.

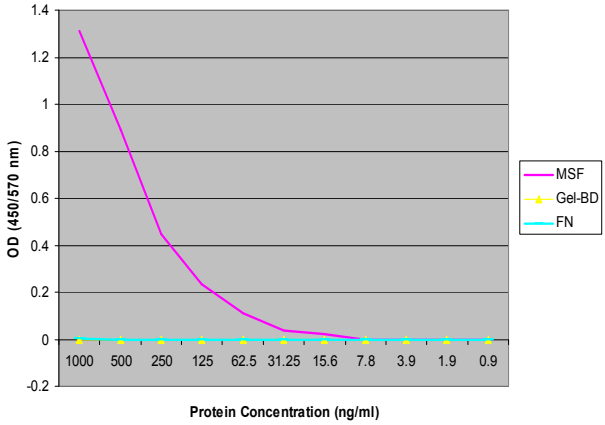
3.4.3.1 Unique MSF-C-terminal identification antibodies (VSI)

A panel of polyclonal and monoclonal antibodies have been generated against the MSF-unique C-terminal decamer (VSIPPRNLGY). Specificity and cross-reactivity of these VSI antibodies were assessed by ELISA (Fig 3.7a & b), dot-blots (Fig 3.7c).These antibodies recognise MSF and do not cross-react with full-length cellular fibronectin (Fn) or proteolytically derived fibronectin fragments, such as Gel-BD (Fig. 3.7b).

(a) Indirect ELISA with (VSI Ab)



(b) Sandwich ELISA with Mab 7.1(VSI Ab)



(c) Dot blots

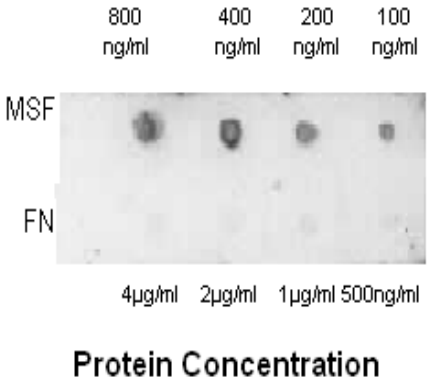


Figure 3.7: Recognition of MSF and Fn proteolytic fragments by VSI antibody.
(a) MSF standard curve by indirect ELISA (Aljorani). There was no detectable cross-reactivity with FN or its proteolysis fragments by sandwich ELISA (b) (Dr. Kankova) or dot blots (c) (Dr. Ellis).

3.4.3.2 MSF-aa identification antibodies (TYN)

TYN (1.1 and 1.2) monoclonal antibodies were raised against the peptide TYNDRTDSTTSNY, an amino acid sequence that is present in MSF-aa only. TYN antibodies were tested against rhMSF-aa, rhMSF+aa, cFn and GBD by both indirect and sandwich ELISA (Dr S.Jones). The results showed good standard curves with rhMSF-aa and no cross reactivity with rhMSF+aa, cFn and GBD (Table. 3.4).

Table 3.4: Standard curve for rhMSF-aa and negative controls by sandwich ELISA

Antibody	Antigen	Concentration ng/ml	Reading	Blank	Final Reading
TYN 1.2 1µg/ml	MSF-aa	200	3.060	0.256	2.804
		100	1.609		1.353
		50	0.963		0.707
		25	0.609		0.353
		12.5	0.448		0.192
		6.25	0.358		0.102
		3.125	0.325		0.069
	MSF+aa	200	0.293	0.256	0.037
	cFn	1000	0.261		0.005
	GBD	1000	0.247		0.009

3.4.3.3 Function-neutralising monoclonal antibodies (PEPQ)

Function-neutralising antibodies (PEPQ) recognise the IGDQ sequence in module 7FnI (Schor *et al.,* 2003; Jones et al., 2007 and Ellis *et al.,* 2010). The following proteolytic fragments of Fn and related proteins had been tested for cross-reactivity with these antibodies: (i) 30kDa Fn fragment containing only heparin-binding domain 1 (HBD), (ii) 45kDa fragment containing only gel-binding domain (GBD) (iii) rhMSF and (iv) full-length human plasma Fn. Results were in agreement with the theoretically predicted specificity, HBD and full-length Fn are recognized neither by PEPQ nor by VSI antibodies. The 45kDa GBD is recognized by PEPQ but not VSI Ab (Fig. 3.8).

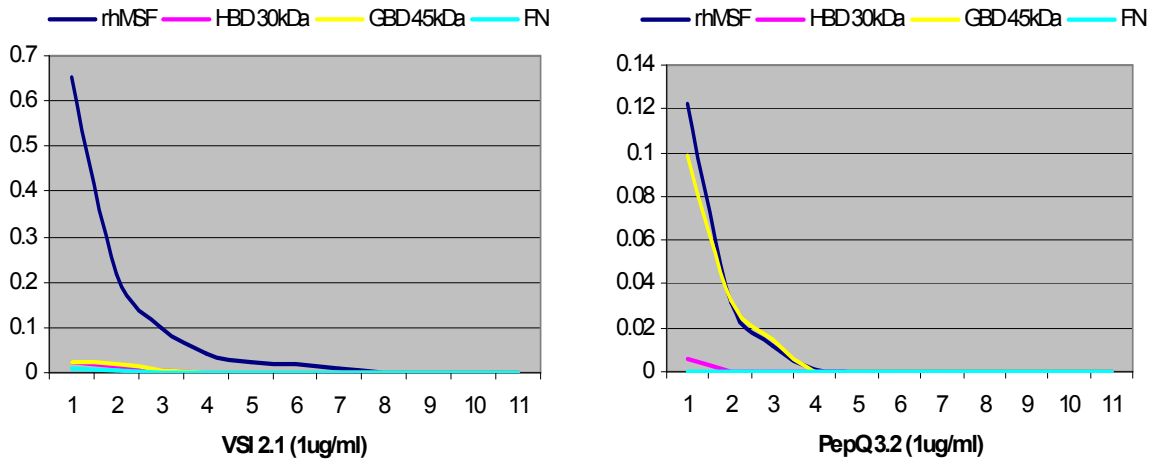


Figure 3.8: Recognition of Fn and its proteolytic fragments by VSI and PEPQ antibodies by indirect ELISA. (Schor et al., 2003)

3.4.4 Inhibition of MSF bioactivity by antibodies and other proteins

3.4.4.1 The effect of MSF-function-neutralising antibodies (PEPQ)

MSF bioactivities can be abrogated by the function-neutralising anti-MSF antibodies (PEPQ 1.1). Exposure of target cells to PEPQ 1.1 did not affect the control or baseline migration, it also had no stimulatory effect on cell migration (TYS, HSG and Endo 742) when tested on its own at 1ng/ml-1µg/ml. However, different concentrations of antibody (10pg/ml–1µg/ml) effectively neutralised the motogenic activity of 100pg/ml of rhMSF. The addition of PEPQ 1.1 together with rhMSF at 100 pg/ml, reduced the number of cells migrated from values comparable to those of positive controls (100pg/ml rhMSF+aa) to baseline levels (Fig 3.9 & 3.10). The addition of PEPQ also abrogated the stimulatory effect of rhMSF+7,9mHm on the migration of Endo 742 cells (Fig 3.11).

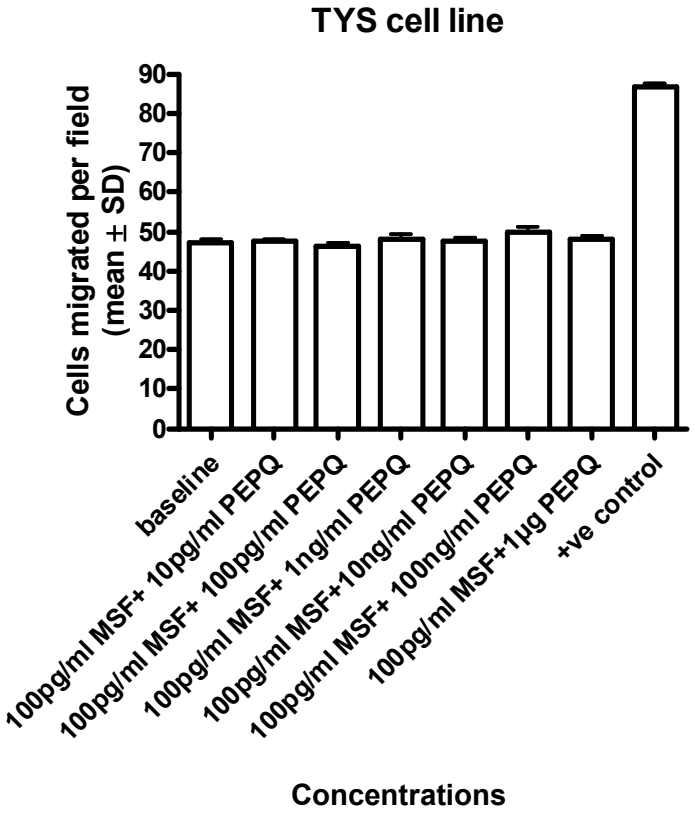


Figure 3.9: Effects of PEPQ antibody on the migration of TYS cells in the transmembrane migration assay.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa was used as a positive control. MSF function-neutralising antibody inhibited the motogenic activity of rhMSF+aa down to the level of the negative control (Bonferroni's test).

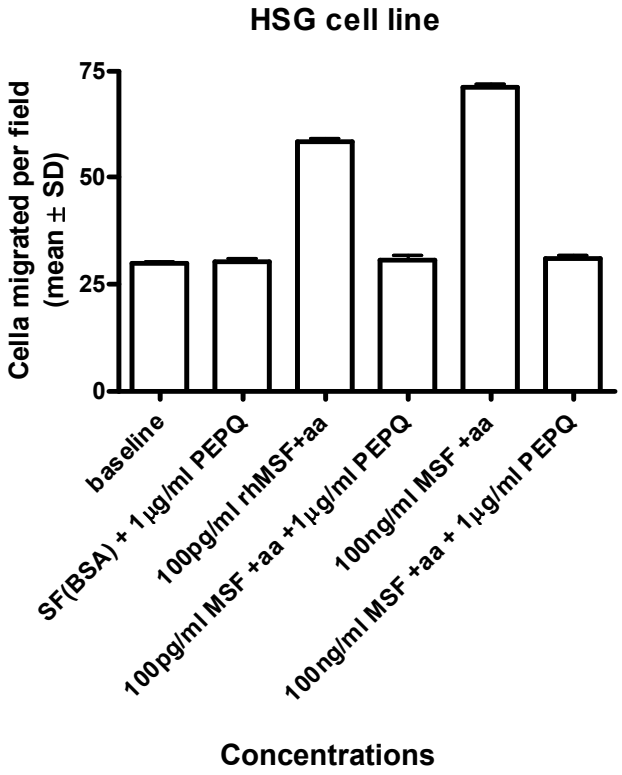


Figure 3.10: Effects of PEPQ antibody on the migration of HSG cells in the transmembran migration assay.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml and 100ng/ml rhMSF+aa as a positive control. MSF function-neutralising antibody inhibited the motogenic activity of rhMSF down to the level of the negative control (Bonferroni's test).

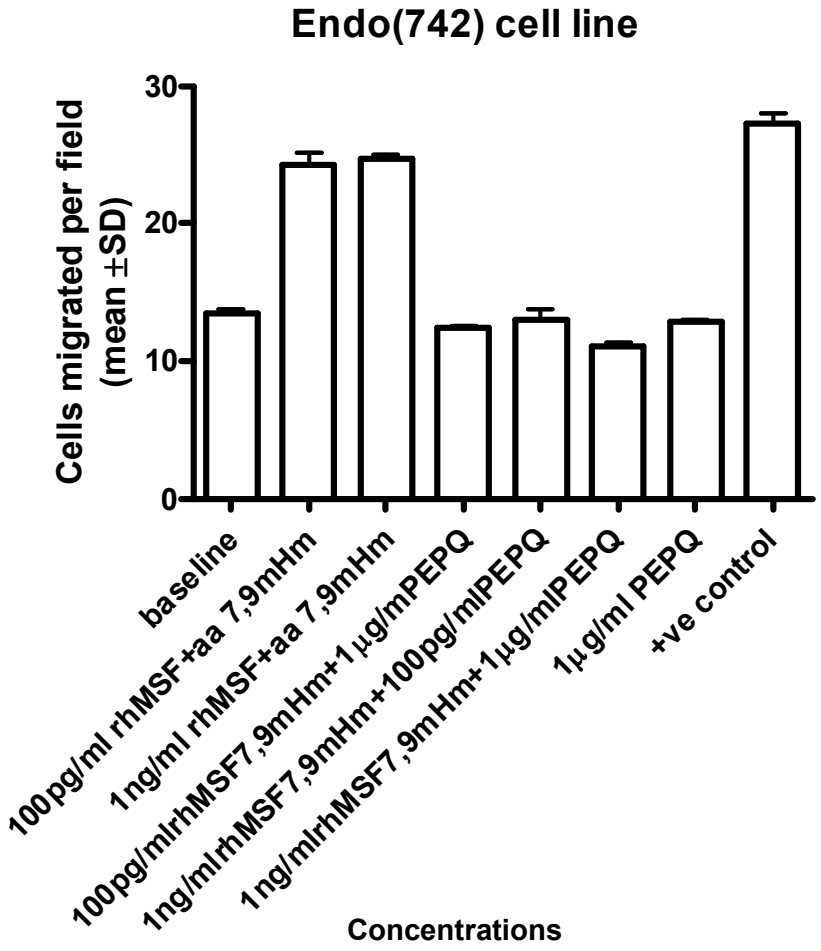


Figure 3.11: Effects of PEPQ antibody and MSF 7,9mHm on the migration of Endo 742 cells in the transmembrane migratiion assay.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. MSF function-neutralising antibody inhibited the motogenic activity of rhMSF 7,9mHm down to the level of the negative control (Bonferroni's $P>0.05$ against baseline), whilst a significant effect of rhMSF 7,9m Hm on the migration was observed in comparison to the negative control (Bonferroni's $P<0.05$).

3.4.4.2 The effect of anti MSF-aa antibody (TYN)

Exposure of target cells (TYS and Endo 742) to antibody TYN1.2 did not affect the control or baseline migration and also had no stimulatory effect on cell migration when tested on its own at 1µg/ml and had no effect on the motogenic activity when added together with rhMSF-aa at 100pg/ml and 1ng/ml. (Fig. 3.12). This finding is similar to the VSI identification antibody result by Schor *et al*, (2003).

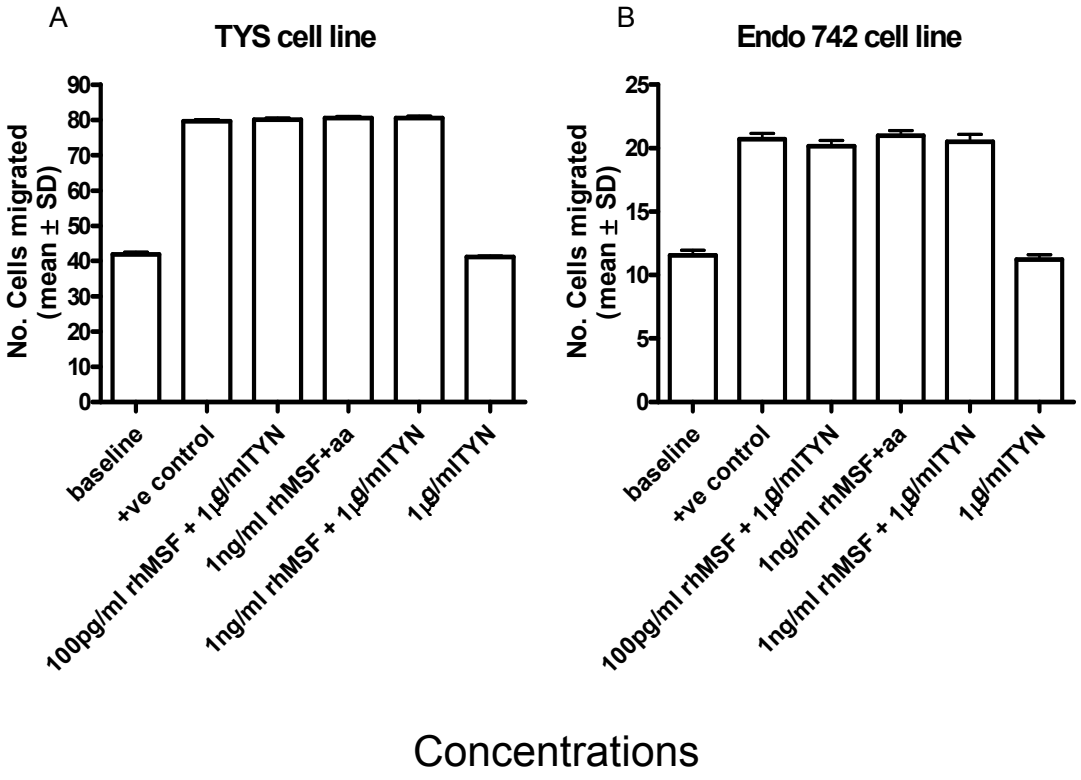


Figure 3.12: Effects of TYN antibody on the migration of TYS and Endo 742 cells in the transmembrane migration assay.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. No significant effects of TYN antibody on the migration of both cells were shown neither alone nor in combination with rhMSF-aa when compared to the negative control (Bonferroni's $P>0.05$).

3.4.4.4 The effect of Lipocalin (NGAL)

The stimulation of target cell migration by MSF is also abrogated by various soluble factors, including the MSF inhibitor NGAL (Jones *et al.,* 2003). Recombinant human NGAL was tested in the transmembrane migration assay at a range of concentrations (1ng/ml, 10ng/ml, and 100ng/ml) in the presence or absence of a single concentration of rhMSF+aa and rhMSF-aa at (100pg/ml). rhNGAL did not affect the baseline level of migration when tested on its own. The chemotactic effect of 100pg/ml rhMSF+aa on TYS (Fig. 3.13) and HSG cells (Fig. 3.33) was completely neutralised by rhNGAL at all above concentrations to the baseline levels, whereas the stimulation of TYS, Endo 742 and HSG migration by 100pg/ml rhMSF-aa was not affected by NGAL at all the above concentrations (Fig. 3.14 & 3.15 & 3.16).

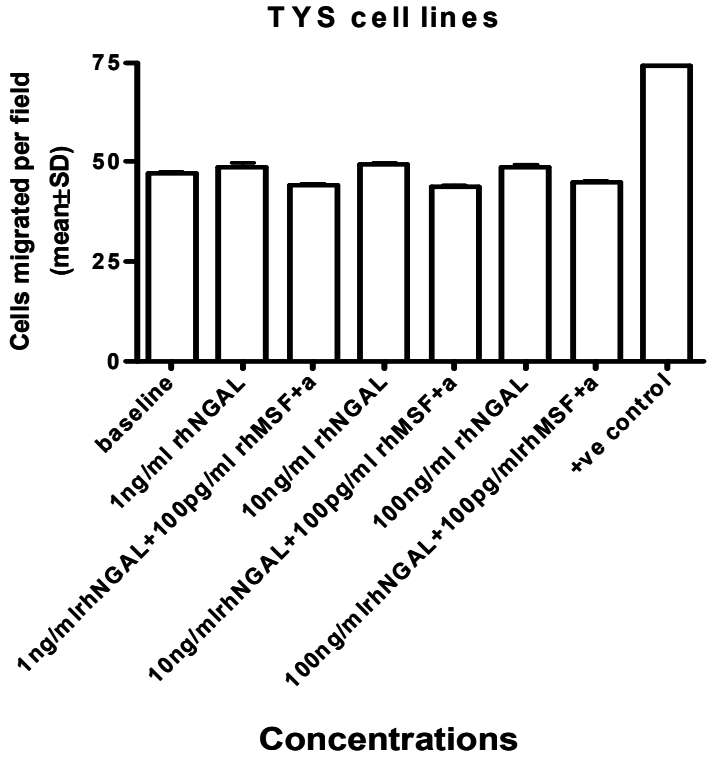


Figure 3.13: Effects of different concentrations of rhNGAL on the migration of TYS cells in the transmembrane migration assay.
A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. No significant effect of rhNGAL on the migration was shown in comparison to the negative control. Whilst, addition of rhNGAL and rhMSF +aa reduced the migration of TYS cells to baseline levels (Bonferroni's $P<0.05$).

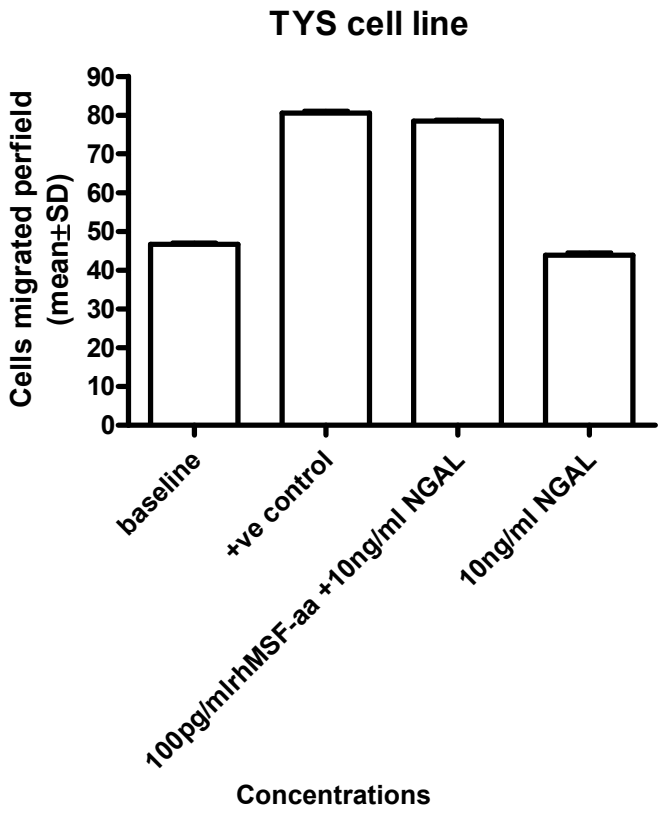


Figure 3.14: Effects of different concentrations of rhNGAL on the motogenic activity of rhMSF-aa on TYS cells in the transmembran migration assay.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF-aa as a positive control. NGAL alone did not affect the baseline migration. Addition of rhNGAL did not affect the motogenic activity of rhMSF-aa on the migration of TYS cells (Bonferroni's test).

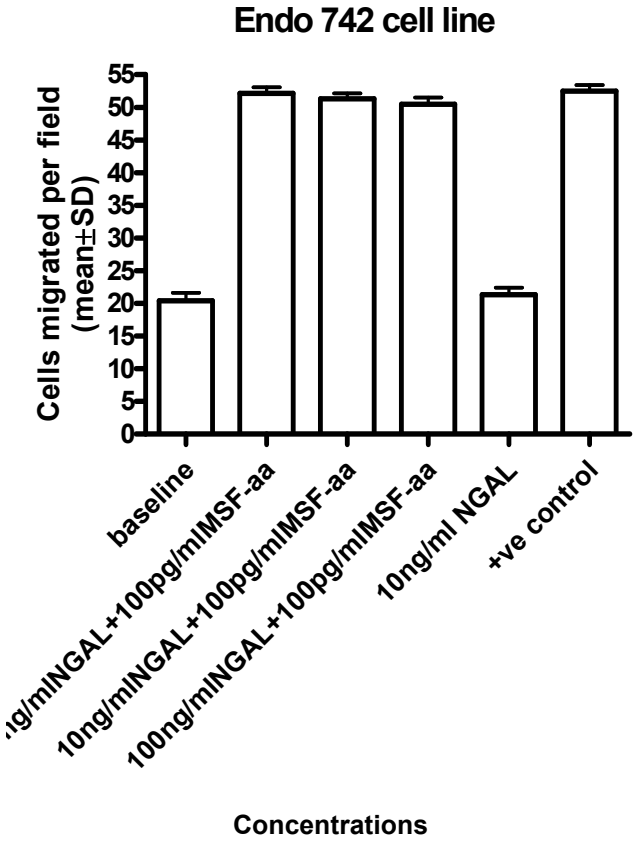


Figure 3.15: Effects of different concentrations of rhNGAL on the motogenic activity of rhMSF-aa on Endo 742 cells in the transmembrane migration assay.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF-aa as a positive control. rhNGAL alone did not affect the baseline migration. Addition of NGAL did not affect the motogenic activity of rhMSF-aa on the migration of Endo 742 cells (Bonferroni's $P>0.05$).

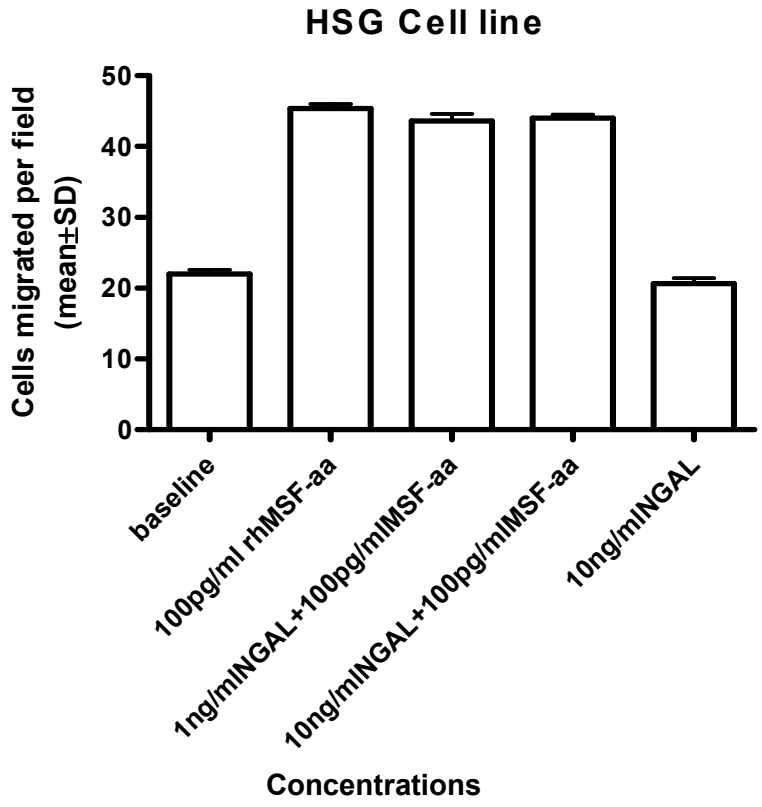


Figure 3.16: Effect of different concentrations of rhNGAL on the motogenic activity of rhMSF-aa on HSG cells in the transmembrane migration assay.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF-aa as a positive control. NGAL alone did not affect the baseline migration. Addition of rhNGAL did not affect the motogenic activity of rhMSF-aa on the migration of HSG cells (Bonferroni's $P>0.05$).

3.4.4.5 The effect of Insulin-like growth factor binding protein-7 (IGFBP-7).

Recent data obtained in the CMB laboratory (Schor, Jones and Florence unpublished; personal communication by Dr Ana Schor) has indicated that IGFBP-7 is an inhibitor of MSF+aa-stimulated fibroblast migration. The effects of recombinant human rhBP-7 on the migration of TYS and Endo742 cells were tested. rhBP-7 was tested at concentrations of 10ng/ml, 100ng/ml and 1µg/ml, on its own and in combination with MSF+aa and MSF-aa at 100pg/ml. When both compounds were added together, the chemotactic effects of MSF on TYS (Fig. 3.17A and 3.18) and Endo 742 cells were completely neutralised by rhIGFBP at all above concentrations (Fig. 3.17B).

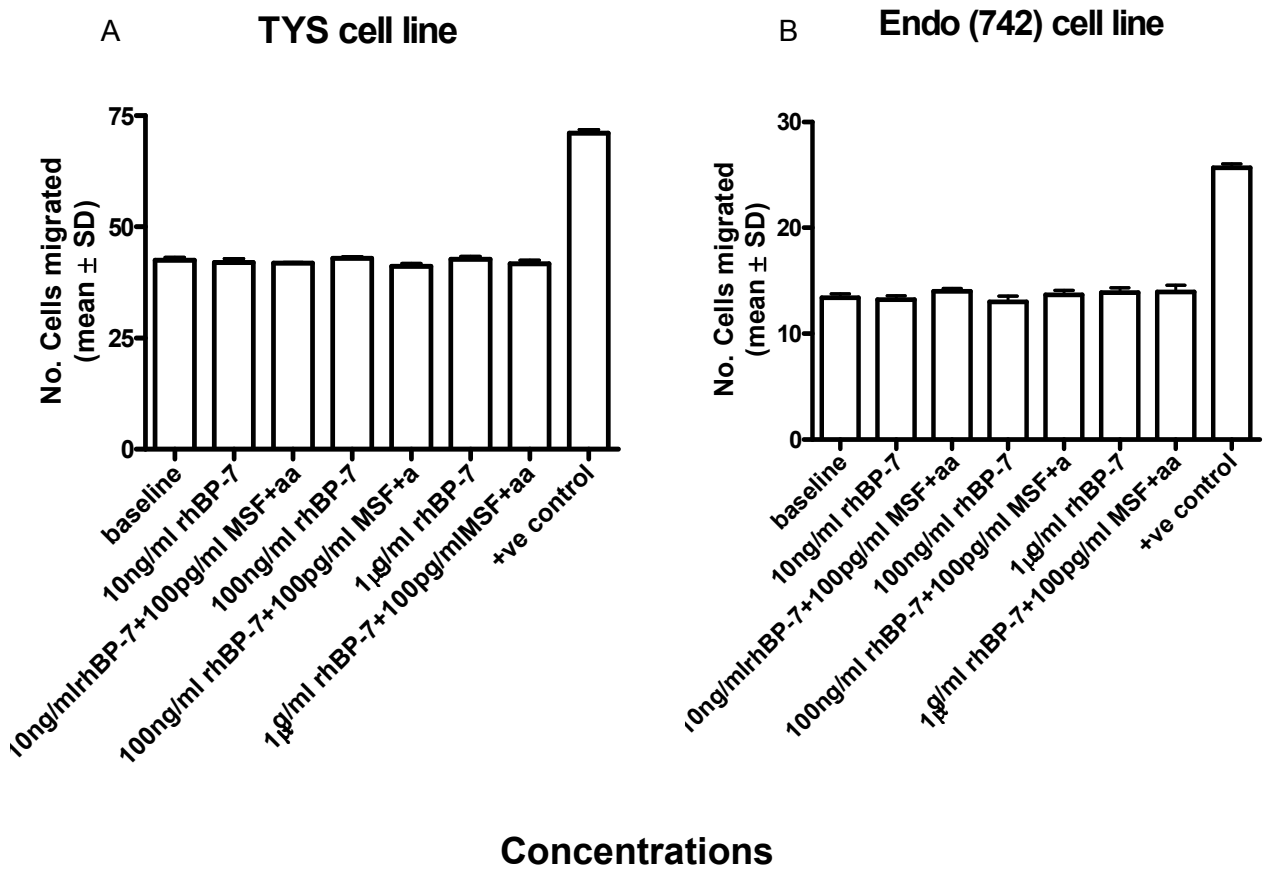


Figure 3.17: Effects of different concentrations rhBP-7 on the migration of TYS (A) and Endo (742) cells (B) in the transmembrane migration assay.
A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. No significant effect of rhBP-7 on the migration was shown in comparison to the negative control (Bonferroni's $P<0.05$). Addition of rhBP-7 inhibited the motogenic activity of rhMSF+aa on the migration of both cells to the baseline levels.

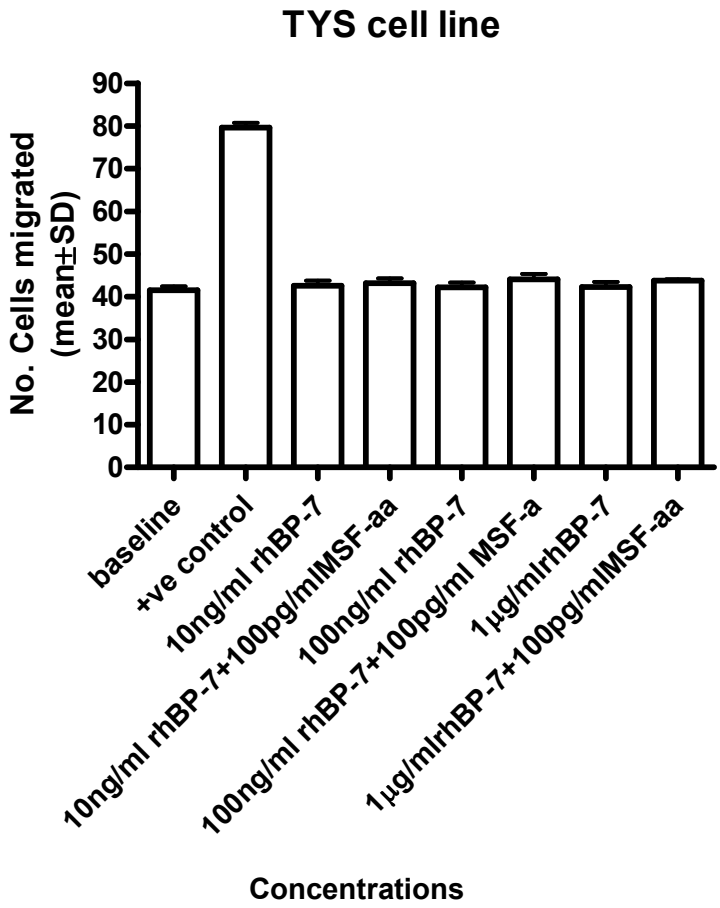


Figure 3.18: Effects of different concentrations of rhBP-7 on the migration of TYS cells in the transmembrane migration assay.

A baseline SF-MEM (containing 2µg /ml bovine serum albumin)was used as a negative control and 100pg/ml rhMSF-aa as a positive control. No significant effect of rhBP-7 on the migration was shown in comparison to the negative control (Bonferroni's $P<0.05$.). Addition of rhBP-7 inhibited the motogenic activity of rhMSF-aa on the migration of TYS cells to the baseline levels.

3.4.5 The production of MSF by tumour cell lines

3.4.5.1. MSF identification by ICC

The expression and the presence of MSF in the TYS and HSG cells were first examined by immunocytochemistry (ICC). TYS cells were plated either within collagen gels or on plastic tissue culture dishes as described in Chapter two (Materials and Methods). Briefly, cells were plated at high density (2 x 10^6 cells/2 ml gel) within and on the surface of 3D collagen gels. The gels were maintained under standard tissue culture conditions for 2 days and then pelleted by gentle centrifugation, formalin fixed and paraffin-embedded. Paraffin-embedded blocks were sectioned and stained with monoclonal MSF-specific identification antibodies mab 7.1 and mab TYN 1.2 as described in Chapter two.

TYS cells were also plated onto plastic surfaces namely 35mm dishes, chamber slides and 24 well plates (Nunclon) at densities ranging from 5-9 x $10^4/cm^2$, 2.2 x10^4 /cm^2 and 2.0 x 10^5 /well respectively. The cells were fixed with 2% (v/v) formalin two days later. Then standard IHC procedures were used to stain with two antibodies mab7.1 and mabTYN 1.2 as outlined in Chapter two.

Data presented in (Fig. 3.19A & B) shows that TYS cells stained positively with monoclonal MSF-specific identification antibodies mab 7.1 and mab TYN 1.2. The same results were obtained irrespective of whether the cells were embedded into collagen gels, paraffin-embedded and sectioned or stained directly on tissue culture dishes, (Fig 3.19G), negative controls, incubated with normal mouse IgG, showed no staining. Identical results were obtained using the salivary gland tumour cell line HSG (Fig 3.19F & H).

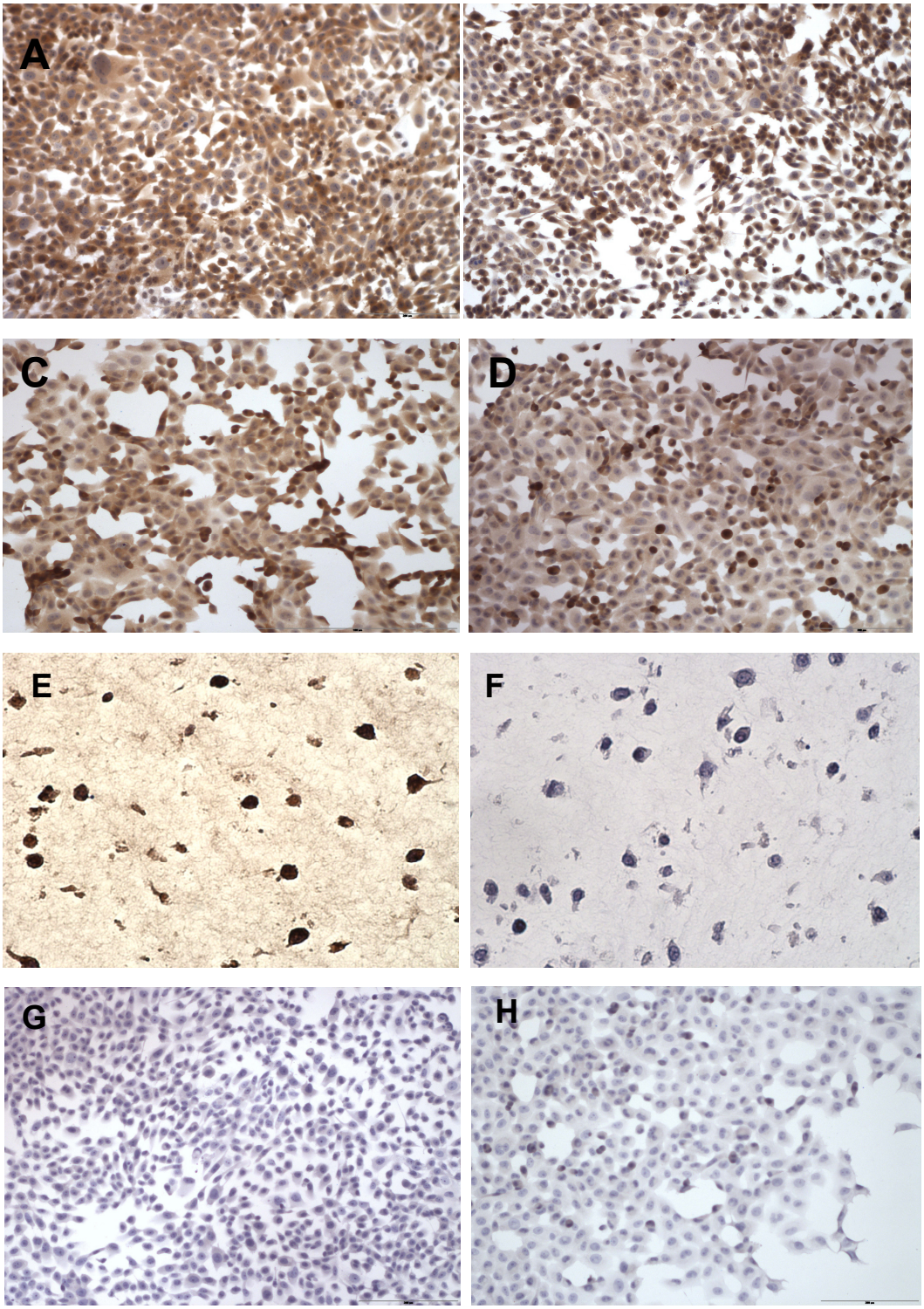


Figure 3.19: Expreesion of MSF by TYS and HSG cells.
Immunostaining with antibodies 7.1 (for total MSF) and TYN (for MSF-aa) (A) TYS cells on tissue culture dishes; positive staining with Ab 7.1. (B) Positive MSF-aa staining in TYS cells on tissue culture dishes by Ab TYN. (C) Positive total MSF staining in HSG cells on tissue culture dishes by Ab 7.1. (D) Positive MSF-aa staining in HSG cells on tissue culture dishes by Ab TYN. (E) Positive MSF staining in HSG cells embedded into collagen gels. (F, H) Negative control stained (HSG) and (G) in TYS with normal mouse IgG. Original photographs were taken at magnification x100 (A, B, C, D, G, H) or x 400 (E, F).

3.4.5.2. MSF identification in cell lysates

An affinity chromatography column was prepared by binding MSF-specific rabbit polyclonal antibody (RpVSI) to reactigel resin (Pierce CDI- Agarose). TYS cells and FSF44 fibroblasts were lysed; the cell lysates were then incubated with resin-bound MSF-specific antibody and the bound material was then eluted with 20mM Tris HCl + NaCl 2M pH 7.4. The cell lysates and resultant fractions (unbound, bound) were tested to identify MSF by using indirect ELISA and Western blots. The affinity chromatography methods used are described in chapter two (Materials and Methods).

Indirect ELISA was performed using a specific anti-MSF antibody (Monoclonal VSI antibody). Unknown samples were compared with a standard curve for rhMSF (0.78ng/ml - 50ng/ml) (Fig 3.20, Table 3.5) and the concentration of MSF present in the unknown samples was estimated by linear regression and Fit spline tests. The results indicated the presence of MSF in the whole TYS cell lysate and in the fraction of TYS lysate that was bound and then eluted from the column (E-TYS-VSI), but not in the unbound fraction. On the other hand, MSF was not present in any of the fractions isolated from FSF44 fibroblasts. The concentration of total MSF in the whole TYS cell lysate was 11-13ng/ml (0.5 ml were obtained from 6 x 10^6 cells), the concentration in the eluted TYS sample was 4-6ng/ml (1ml obtained from 3ml whole cell lysate). The sensitivity of this assay was found to be 3ng/ml (Table 3.5).

The Western blot results are shown in (Fig 3.21). It is apparent that TYS cells (eluted fraction and whole lysate) expressed MSF protein, corresponding to an approximate molecular weight of 70 kDa.

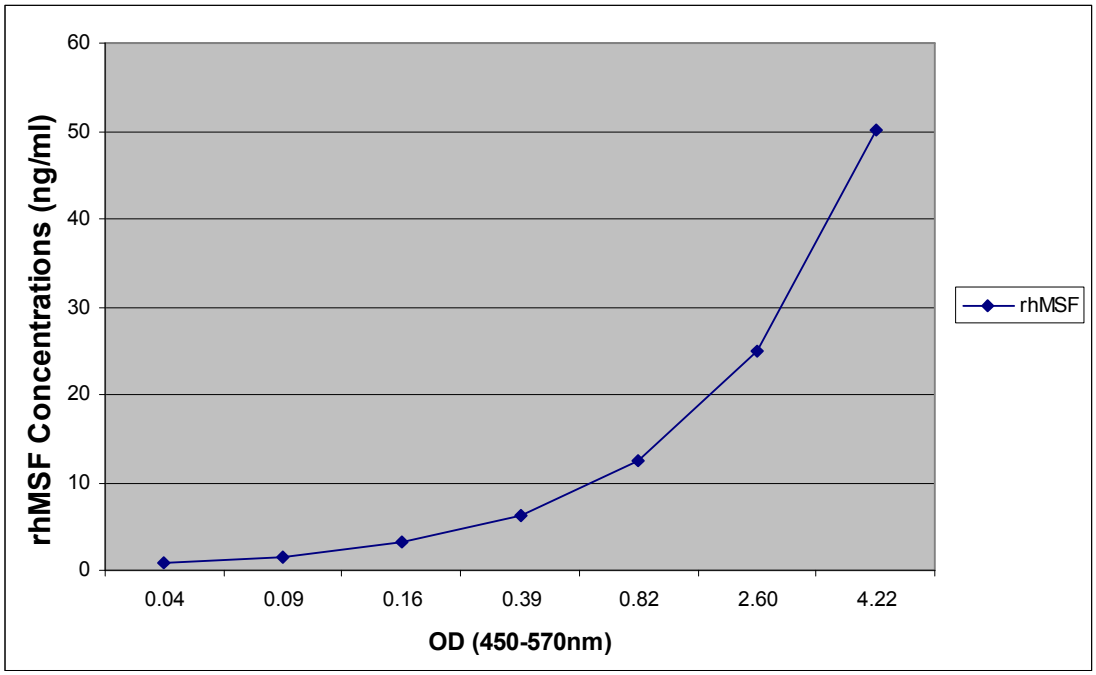


Figure 3.20: Indirect ELISA. rhMSF+aa standard curve.

Table 3.5: Summary of MSF+aa standard curve (concentration / OD 450-570nm) unknown fractions (sample / OD 450-570nm) estimated concentrations.

rhMSF standard curve		**unknown fractions**			
Concentration in ng/ml	**OD**	**sample**	**OD**	**estimated concentration**	
				Fit/spline test	**Linear regression**
50	4.1795±0.05	E-TYS (VSI)	0.345±0.02	6.428ng/ml	4.403 ng/ml
25	2.735±0.195	TYS (whole cell lysate)	0.9355±0.006	12.86 ng/ml	11.015 ng/ml
12.5	0.8925±0.01	FSF44 fraction	0.0205±0.03	0	0.686 ng/ml
6.25	0.3415±0.06	Unbound fraction-TYS (VSI)	0.069±0.003	1.4ng/ml	1.233 ng/ml
3.125	0.152±0.008	Sensitivity: SD of blank x 3= 0.048 x 3= 0.144 ~ 3 ng/ml **Fit/spline** ~ 2.08 ng/ml **Linear regression**			
1.56	0.0785±0.02				
0.58	0.04± 0				
0 (PBS) blank	0.048±0.048				

E-TYS (VSI) = eluted fraction of TYS sample across VSI affinity column, FSF44 fraction= the fractions isolated from FSF44 fibroblasts

The table shows the concentrations of Total MSF present in the whole TYS cells, unbound proteins, RpVSI column eluted fractions.

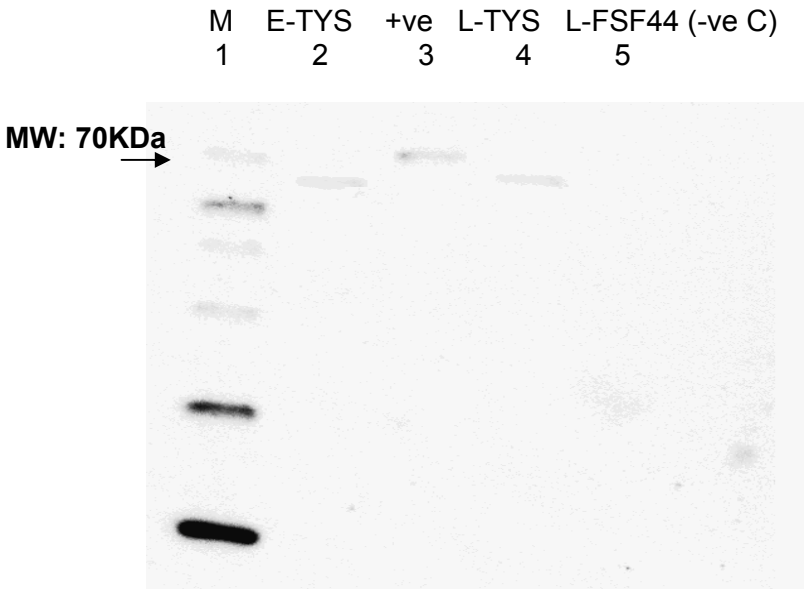


Figure 3.21: Identification of MSF in TYS cell lysates.
Lane1(Magic marker), lane 2 correspond to the eluted fraction E-TYS (VSI), lane 3 positive control (500ng/ml MSF), lane 4 protein from whole TYS cell lysates and lane 5 the negative control (FSF44 cell lysate).

3.4.5.3 MSF identification by the motogenic activity present in conditioned medium

3.4.5.3.1 Motogenic activity present in the CM of TYS and HSG cells

In order to ascertain whether the motogenic activity produced by oral tumour cell line TYS involved MSF, SF-CM from TYS cells (TYS-CM) was obtained and stored as described in Chapter two (Materials and Methods).

The potential motogenic activity of the TYS-CM was tested at various dilutions in the transmembrane migration assay using TYS as target cells. TYS- CM was diluted (1:2, 1:4, 1:10 and 1:20) in serum-free- MEM containing 2µg/ml BSA. The chamber was prepared and cells added as described in Chapter two. Cell migration was determined after a 5 hour incubation period. Experiments were repeated 3-4 times. Consistent stimulation of TYS migration was observed with TYS-CM at a range of dilutions from neat to 1:10 (Fig 3.22).

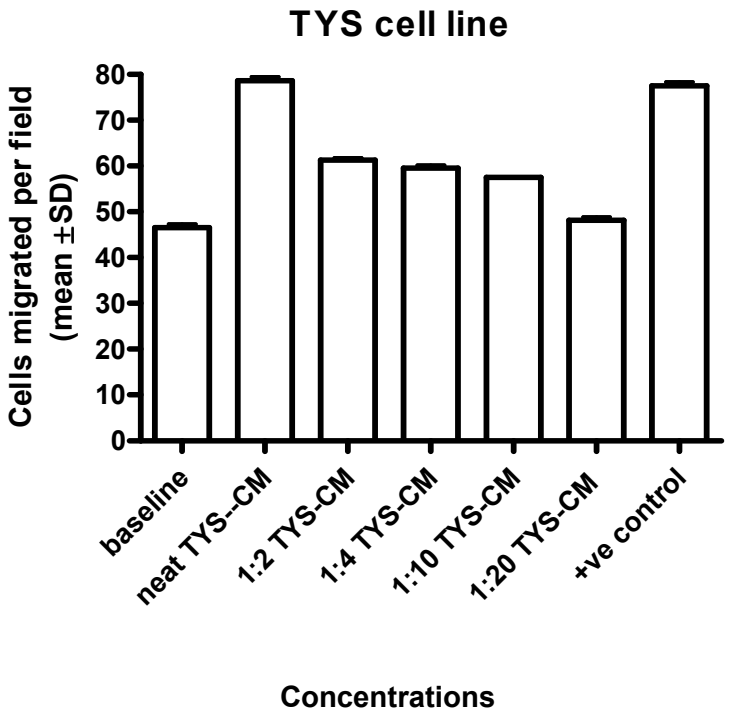


Figure 3.22: Motogenic effects of TYS-CM on TYS cells.
SF-MEM containing 2µg/ml BSA was used as a negative control (baseline) and. 100pg/ml rhMSF+aa as a positive control. Data showed that TYS-CM (neat-1:10) had significant stimulatory effect on the migration of TYS cells by comparison to the negative control (Bonferroni's $P<0.05$). No significant effect was shown at 1:20 dilution.

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HSG-CM:

The chemotactic activity of conditioned medium from HSG cells in promoting their own migration was ascertained as described above for TYS cells. Using the transmembrane migration assay, HSG-CM was diluted (1:2) in serum-free- MEM containing 2µg/ml BSA. The chamber was prepared and cells added as described in Materials and Methods. Experiments were repeated 2 times. Consistent stimulation of HSG migration was observed with HSG-CM at both concentrations tested (neat and 1:2 dilution) (Fig 3.23).

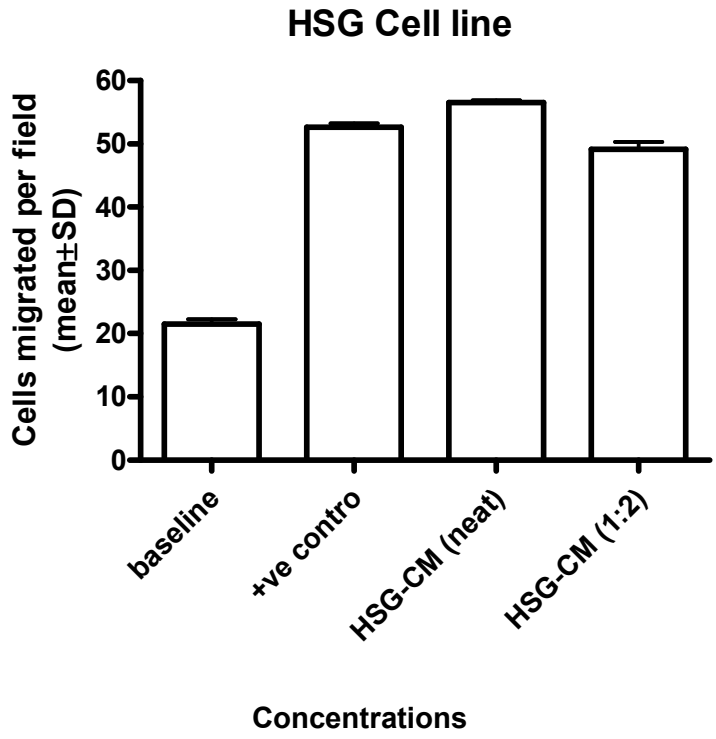


Figure 3.23: Motogenic effects of HSG-CM on HSG cells.
SF-MEM containing 2µg/ml BSA was used as a negative control (baseline) and. 100pg/ml rhMSF+aa as a positive control. Data showed that (HSG-CM) (neat and 1:2 dilution had a significant stimulatory effect on the migration of HSG cells by comparison to the negative control (Bonferroni's $P<0.05$).

3.4.5.3.2 Inhibition of CM motogenic activity by MSF-function-neutralising antibodies (PEPQ)

Identification of MSF bioactivity in TYS-CM was tested using the transmembrane assay and anti-MSF function-neutralising antibodies (PEPQ). Addition of antibody PEPQ at 1ng/ml, 10ng/ml, and 100ng/ml reduced the TYS-CM-stimulated migration of TYS cells to or under the baseline levels. The antibody by itself had no effect on migration (Fig 3.24).

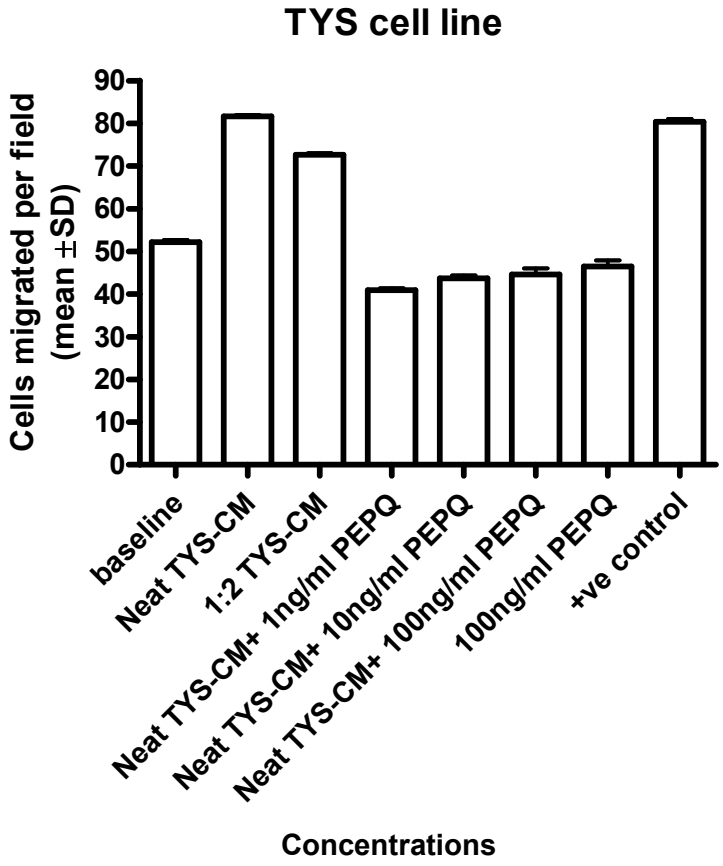


Figure 3.24: Neutralisation of TYS-CM motogenic activity by MSF-function neutralising Ab (PEPQ). Effect on target TYS cells

TYS-CM (neat and 1:2 dilution) significantly stimulated the migration of TYS cells (Bonferroni's $P<0.05$, relative to the baseline). Addition of Ab PEPQ at different concentrations, together with TYS-CM reduced cell migration to baseline levels. Negative and positive controls were as in previous graphs.

3.4.5.3.3 Binding of CM motogenic activity to MSF identification antibodies (VSI)

MSF was identified in the oral tumour cell line (TYS) conditioned medium (TYS-CM) using affinity chromatography as described for cell lysates (3.4.5.2) and in chapter two. TYS-CM was incubated with the MSF-specific antibody (RpVSI) column and the bound material was eluted with 2M NaCl + 20mM Tris HCl pH 7.4. The resulting fractions are referred to as unbound (U-[TYS CM]-VSI) or bound and eluted (E-[TYS CM]-VSI). The various fractions were tested for motogenic activity in the transmembrane assay, using TYS as a target cells (Figs. 3.25 & 3.26) The results, indicated that the motogenic activity of TYS-CM was due to the presence of MSF, as it was only recovered from the material specifically bound to RpVSI and then eluted from the column (E-[TYS CM]-VSI). Significantly, no motogenic activity was detected in the unbound fraction (Fig 3.25 & 3.26). Motogenic activity was recovered in the first eluted fraction (E1-[TYS CM]-VSI) (Fig 3.26). Fig 3.25 also shows the chemotactic effect of unfractionated TYS-CM, used as positive control, and the lack of activity of the elution buffer, used as an additional negative control. Identical results were obtained using fibroblasts (FSF44) and human endothelial cells (Endo 742) as a target cells (Fig. 3.27 & 3.32).

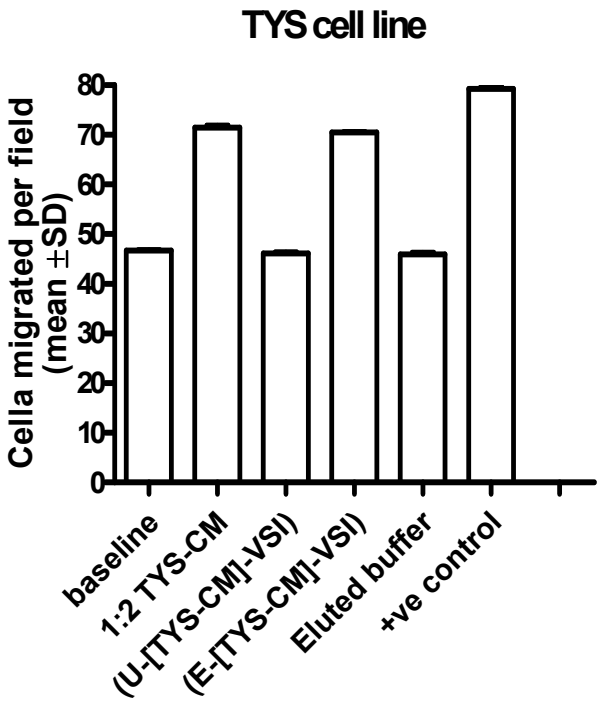


Figure 3.25: Identification of MSF in TYS-CM. Fractions were obtained by affinity chromatography using MSF-specific antibody (RpVSI) column.
The original sample (unfractionated TYS-CM, diluted 1:2), unbound fraction (U-[TYS CM]-VSI) and eluted fraction (E1-[TYS CM]-VSI) were tested for migration stimulating activity in the transmembrane assay. SF-MEM was used for the baseline and 100pg/ml rhMSF as a positive control. Data show that the motogenic activity present in TYS-CM was recovered in the material bound to RpVSI and then eluted. The unbound material and the eluted buffer had no significant effect on the migration of TYS, by comparison to the negative control (Bonferroni's $P<0.05$).

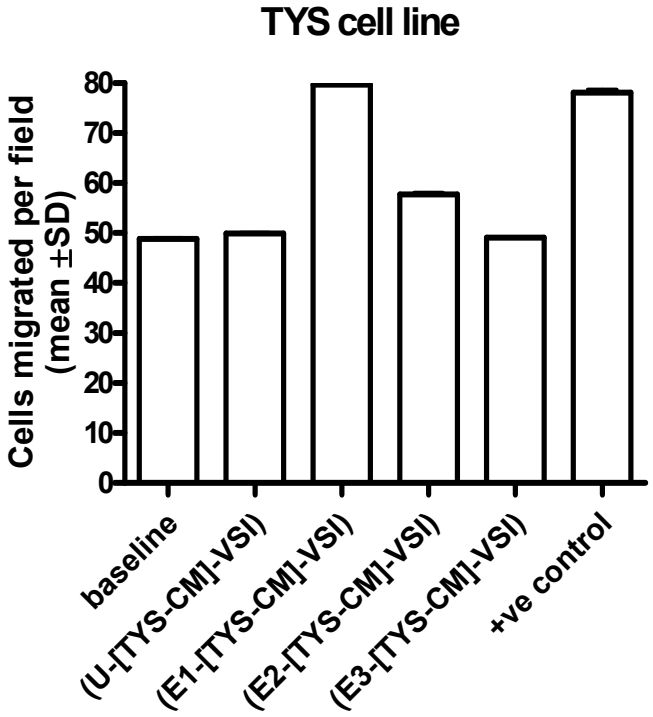


Figure 3.26: Identification of MSF in TYS-CM using affinity chromatography RpVSI column.
The unbound CM and first three eluted samples (E1, E2, and E3) were tested for migration stimulating activity in the transmembrane migration assay. A baseline control level (containing 2µg /ml bovine serum albumin) was used as a negative control was tested and 100pg/ml rhMSF+aa used as a positive control. Data show that the motogenic activity present in TYS-CM was recovered in the first eluted material bound to RpVSI. The unbound (U-[TYS CM]-VSI) and the second and third elutions had no significant effect on the migration of TYS cells (Bonferroni's $P<0.05$).

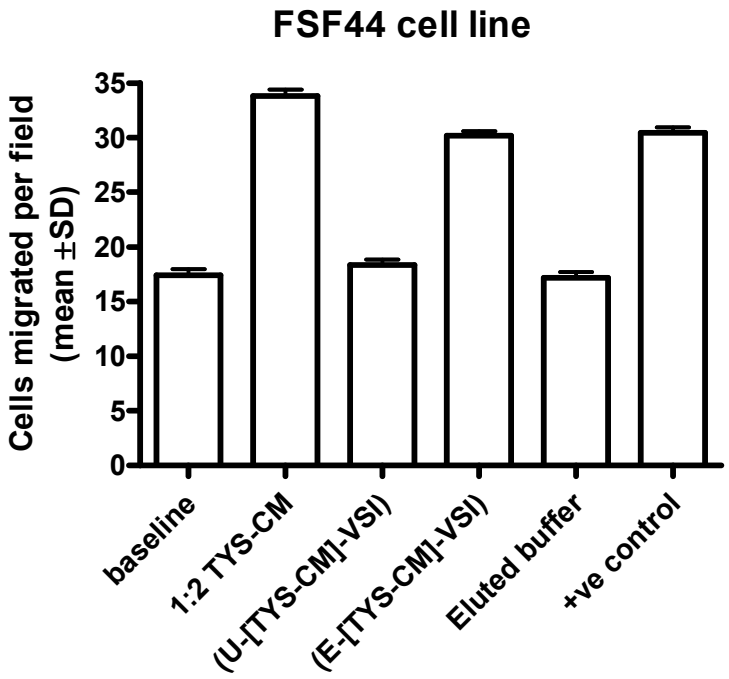


Figure 3.27: Identification of MSF in TYS-CM, after fractionation by RpVSI affinity chromatography column (FSF44).

Fractions were obtained by affinity chromatography as in Figure 3.24 The original sample (unfractionated TYS-CM, diluted 1:2), unbound fraction (U-[TYS CM]-VSI) and eluted fraction (E1-[TYS CM]-VSI) were tested for migration stimulating activity in the transmembrane assay. SF-MEM was used for the baseline and 100pg/ml rhMSF as a positive control. Data show that the motogenic activity present in TYS-CM was recovered in the material bound to RpVSI and then eluted. The unbound material and the eluted buffer had no significant effect on the migration of FSF44, by comparison to the negative control (Bonferroni's $P<0.05$.).

3.4.5.3.4 Binding of CM motogenic activity to MSF-aa identification antibodies (TYN)

MSF-aa was identified in the oral tumour cell line conditioned medium (TYS-CM) using affinity chromatography to an antibody (TYN) that recognises MSF-aa, not MSF+aa. An affinity chromatography column was prepared by binding MSF-aa-specific monoclonal antibody (TYN) to Reactigel resin (Pierce CDI- Agarose). The TYS-CM fraction previously bound to and eluted from the MSF-specific antibody (RpVSI) column (E-[TYS CM]-VSI) (see section 3.4.5.3.3, above) was incubated with the MSF-aa antibody column and the bound material was eluted with 2M NaCl + 20mM Tris HCl pH 7.4. The resulting factions are referred to as unbound (U-[E TYS VSI]-TYN) or bound and then eluted (E-[E TYS VSI]-TYN) The various fractions were tested for motogenic activity in the transmembrane assay, using TYS, FSF44 and HSG as a target cells (Figs. 3.28, 29 & 33) Data show that the motogenic activity was present in both the unbound and the bound fractions, suggesting that the starting material (B-E-VSI) contains MSF+aa (which binds to Ab VSI, but not to Ab TYN) as well as MSF-aa (which binds to both Abs) (Fig 3.28 & 3.29). This hypothesis was tested by examining the effects of NGAL, BP7 and MSF-function-neutralising Ab (PEPQ) on the bioactivity of those fractions containing migration stimulating activity. All these factors inhibit the motogenic activity of MSF+aa, whereas the motogenic activity of MSF-aa is inhibited by BP-7 and Ab PEPQ, but is not inhibited by NGAL.

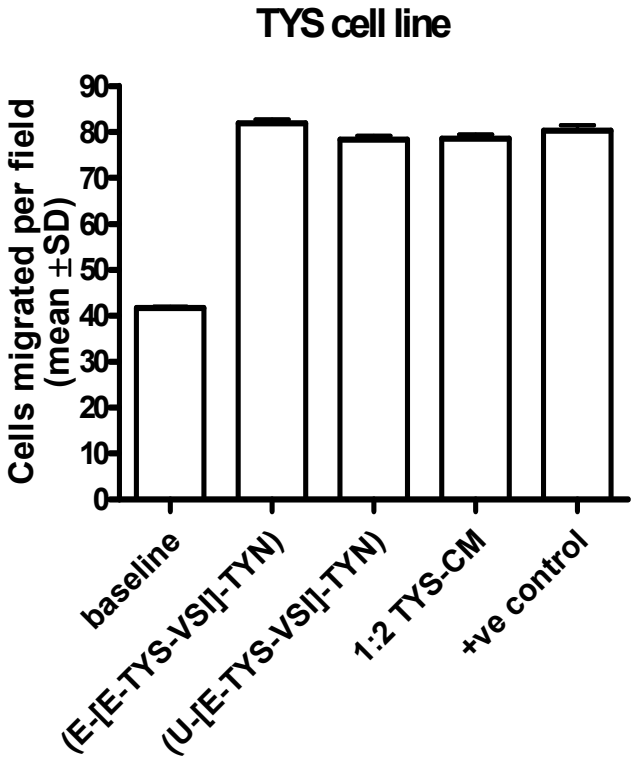


Figure 3.28: Identification of MSF-aa in TYS-CM by TYN affinity chromatography (TYS cell). Fractions were obtained by affinity chromatography as in Figure 24 The original sample (unfractionated TYS-CM, diluted 1:2), unbound fraction (U-[E TYS VSI]-TYN) and eluted fraction (E-[E TYS VSI]-TYN) were tested for migration stimulating activity in the transmembrane assay. SF-MEM was used for the baseline and 100pg/ml rhMSF as a positive control. Data show that the motogenic activity present in TYS-CM was recovered in the material bound to TYN. A significant effect of eluted material sample (MSF-aa), unbound material (expected MSF+aa) and orginal sample (1:2 TYS-CM) on the migration of TYS cells was shown in comparison to the negative control (Bonferroni's $P<0.05$).

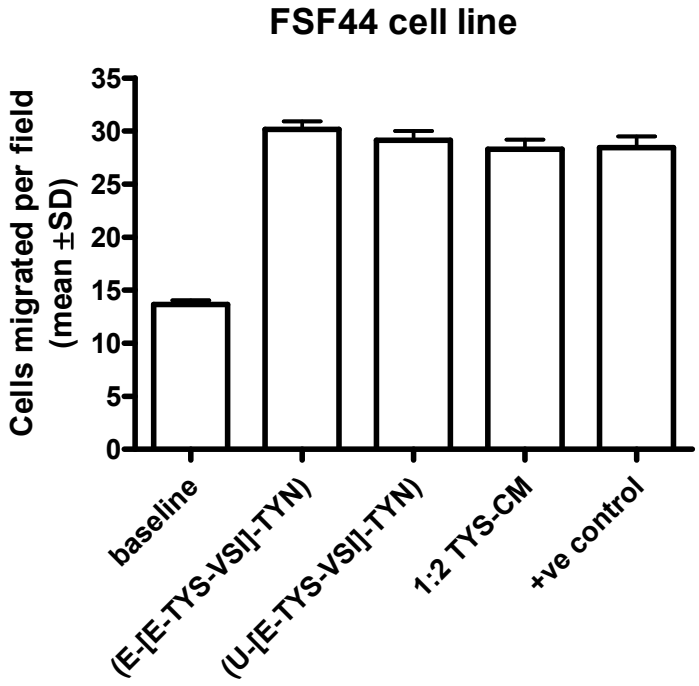


Figure 3.29: Identification of MSF-aa in TYS-CM by TYN affinity chromatography (FSF44).
The fractions containing migration stimulating activity were analysed for the presence of MSF-aa using the transmembrane migration assay. A baseline control level was used as a negative control and 100pg/ml rhMSF+aa as a positive control. Data show that the motogenic activity present in eluted sample was recovered in the material bound to TYN. A significant effect of eluted material sample (MSF-aa), unbound fraction (expected MSF+aa) and orginal sample (1:2 TYS-CM) on the migration of FSF44cells was shown in comparison to the negative control (Bonferroni's $P<0.05$).

Representative results shown in (Figs 3.30, 3.31, 3.32, 3.33 & 3.34) confirmed that TYS-CM, and the motogenic fractions obtained by affinity chromatography (E-VSI and E-TYN) stimulated the migration of target cells TYS and FSF to the same extent as the positive control (rhMSF), The motogenic activities of TYS-CM and E-VSI were inhibited by NGAL, PEPQ and BP-7. In contrast, the motogenic activity of B-E-TYN was inhibited by PEPQ and BP-7 but not by NGAL. The three inhibitory factors tested did not affect baseline migration when tested on their own. The same results were obtained using HSG and Endo 742 as target cells.

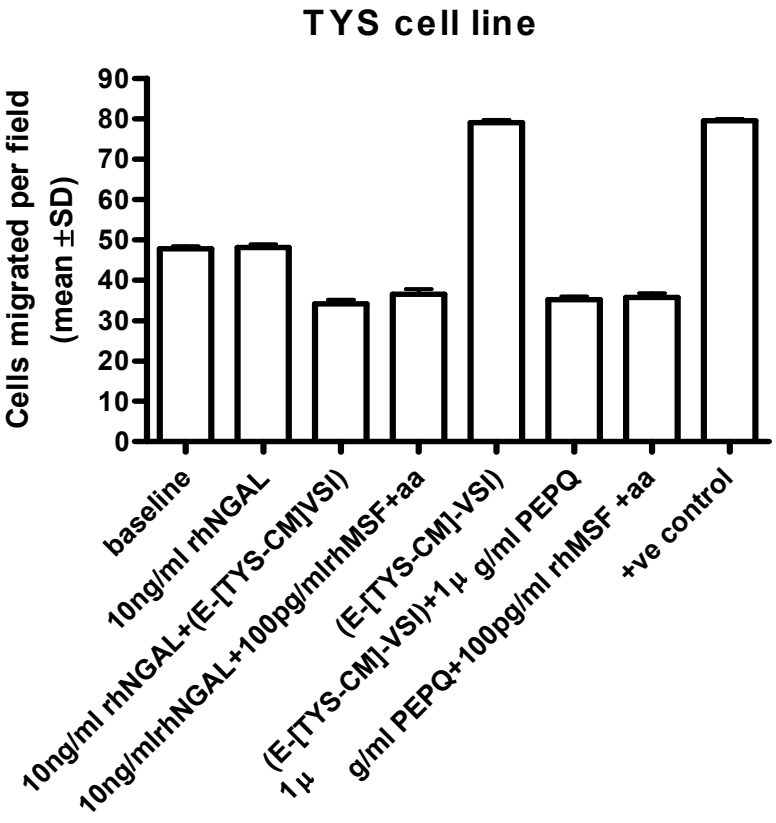


Figure 3.30: Identification of MSF in TYS-CM by RpVSI affinity chromatography (TYS cells). The fractions containing migration stimulating activity was analysed for the presence of MSF using the transmembrane migration assay and an anti-MSF function-neutralising (PEPQ) antibody and rhNGAL. A baseline control level was used as a negative control and 100pg/ml rhMSF+aa as a positive control. Addition of antibody (PEPQ) and rhNGAL reduced the migration of TYS cells to baseline levels. Data show that the motogenic activity present in the CM (MSF) is recovered in the material bound to RpVSI.

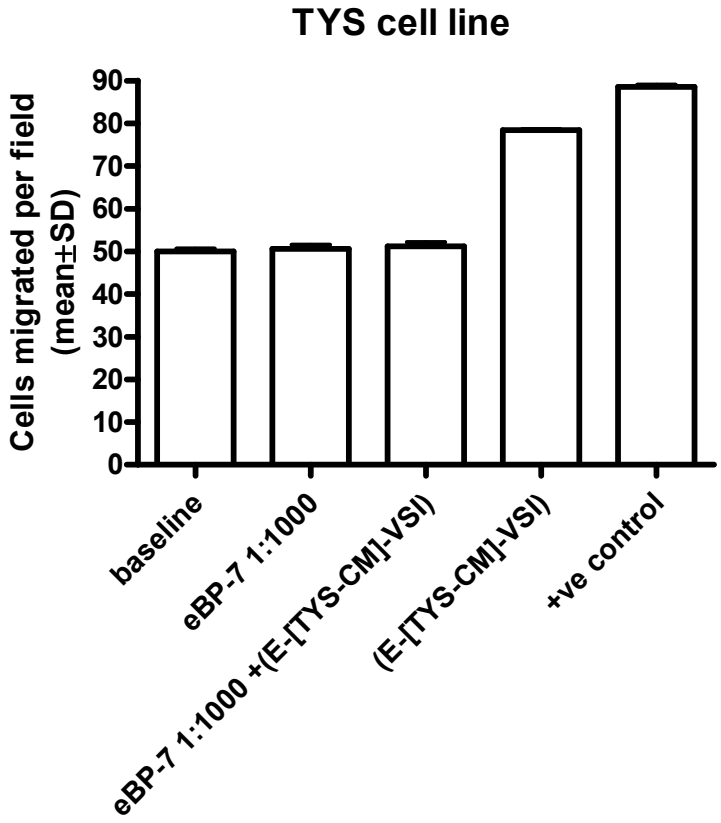


Figure 3.31: Effects of eBP-7 on the motogenic activity of TYS-CM after characterisation by RpVSI affinity chromatography column (TYS cells).

A baseline SF-MEM (containing 2µg /ml bovine serum albumin) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. The motogenic activity of the eluted sample (MSF) was abrogated by the presence of eBP-7 in the migration assay. A significant effect of eluted material sample (MSF) on the migration of TYS cells was shown in comparison to the negative control (Bonferroni's $P<0.05$).

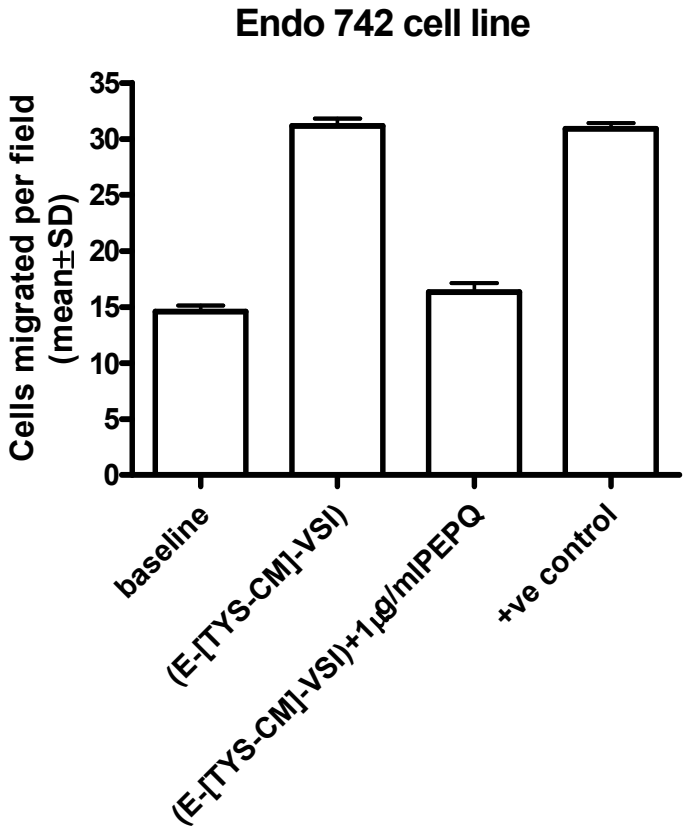


Figure 3.32: Identification of MSF as the induced motogen, after characterisation by RpVSI affinity chromatography column (Endo 742 cells).

The fractions containing migration stimulating activity were analysed for the presence of MSF using the migration assay and specific MSF function-neutralising antibody (PEPQ). A baseline control level was used as a negative control and 100pg/ml rhMSF+aa as a positive control. Addition of Ab PEPQ, together with the eluted sample (MSF), reduced the Endo 742 cell migration to the baseline levels. A significant effect of eluted material sample (MSF) on the migration of Endo 742 cells was shown in comparison to the negative control (Bonferroni's $P<0.05$).

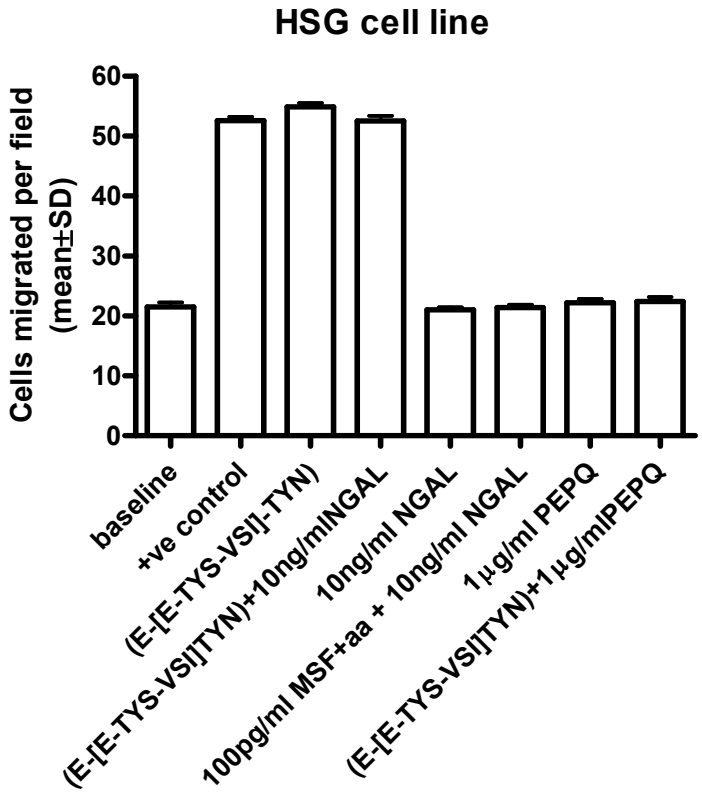


Figure 3.33: Identification of MSF-aa in TYS-CM by TYN affinity chromatography (HSG cells). The fractions containing migration stimulating activity were analysed for the presence of MSF-aa using the transmembrane migration assay and rhNGAL. A baseline control level was used as negative control and 100pg/ml rhMSF+aa as positive control. Addition of rhNGAL to the eluted material (MSF-aa) did not affect the migration of HSG cells, whilst a combination of rhNGAL and MSF+aa reduced the migration to the baseline level. This relates to specificity alone to rhMSF+aa leaving MSF-aa to show its bioactivity in the TYS-CM. Data show that the motogenic activity present in the sample eluted from the RpVSI column is recovered in the material bound to TYN affinity column.

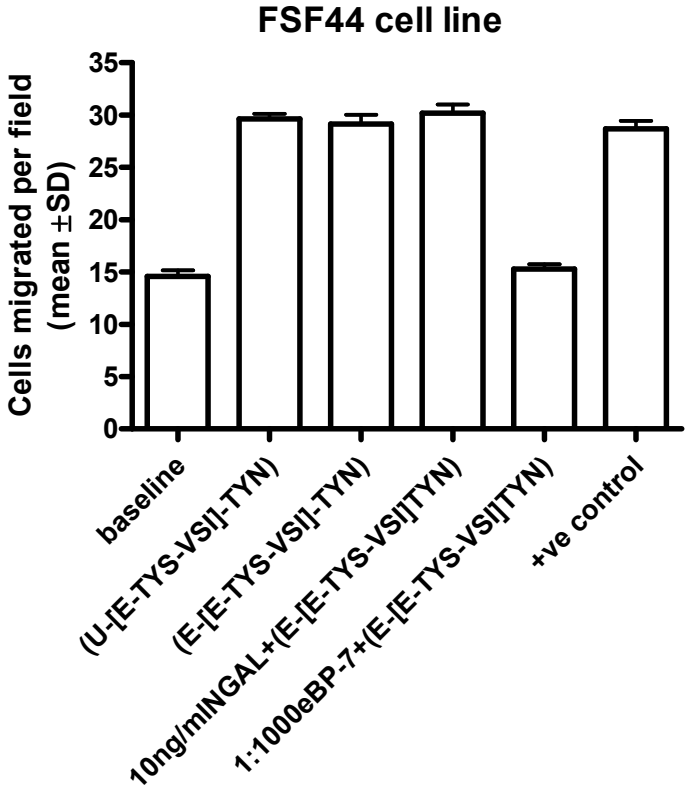


Figure 3.34: Identification of MSF-aa in TYS-CM through TYN affinity chromatography column (FSF44 cells).

The fractions containing migration stimulating activity were analysed for the presence of MSF-aa using the transmembrane migration assay and NGAL and eBP-7. A baseline control level was used as a negative control and 100pg/ml rhMSF-aa as a positive control. Addition of NGAL did not affect on the migration of HSG cells. Whilst, the motogenic activity of eluted bound-TYN (MSF-aa) was abrogated by eBP-7. Data show that the motogenic activity present in the sample eluted from the RpVSI column is recovered in the material bound to TYN.

3.4.6 Possible mechanism of action of the inhibitors of MSF

3.4.6.1 Binding of rhNGAL to MSF

Affinity chromatography techniques as outlined in chapter two (Materials and Methods) and above sections (3.4.5.3.3) were used to investigate whether rhNGAL binds to MSF or there is no direct association. For these experiments, three affinity columns were prepared by binding rhMSF+aa, rhMSF-aa and BSA to Reactigel resin (Pierce CDI- Agarose). rhNGAL (2µg/ml diluted in 20mM Tris HCL pH 7.4) was passed through each of the columns and the bound material was eluted with 2M NaCl + 20mM Tris HCl pH 7.4. This process was repeated 3 times; the eluted samples were pooled, dialysed against distilled water (dH_2O) and then concentrated by freeze drying and redissolved in 0.5ml of PBS. The resultant bound and then eluted fractions are referred to as E-[NGAL]-MSF+aa, E-[NGAL]-MSF-aa and E-[NGAL]-BSA, indicating the affinity column used. These fractions were assayed for the presence of NGAL by indirect MSD ELISA using a specific anti-NGAL antibody (polyclonal antibody AF1757), as described in Chapter two (Materials and Methods). The unknown samples were compared with a standard curve for rhNGAL (0.4ng/ml, 2ng/ml, 10ng/ml and 50ng/ml) and their concentrations estimated by linear regression test (Fig. 3.34). Data in (Table 3.6) shows that NGAL was detected in the sample eluted from the MSF+aa column, but not in the samples eluted from the MSF-aa or the BSA columns. Therefore the results indicate that NGAL binds to MSF+aa and not to MSF-aa. The same results were obtained in three independent experiments.

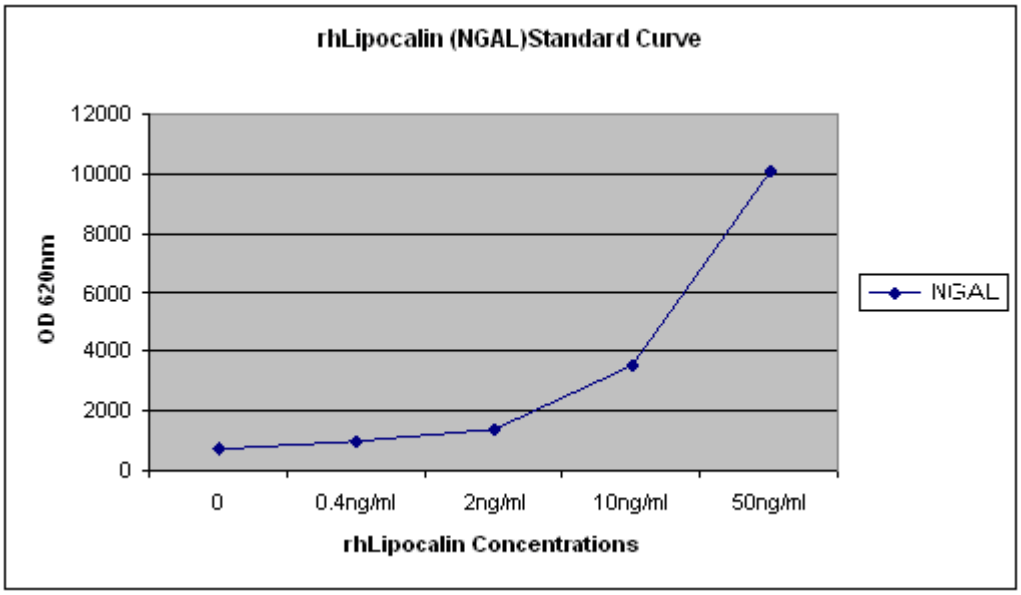


Figure 3.35: Indirect MSD ELISA standard curve of rhNGAL.

Table 3.6: MSD ELISA legend explains Ab and general methods
Concentration of NGAL in samples eluted from different affinity chromatography columns. The concentration was estimated by linear regression.

Sample	Affinity column	OD (620nm) Blank substracted	estimated concentration	Comments
E-[NGAL]-MSF-aa	**MSF-aa**	**- 437.5**	**0**	**undetectable**
E-[NGAL]-MSF+aa	**MSF+aa**	**1381 / 1038**	**1.6-2.0ng/ml**	**detectable**
E-[NGAL]-BSA	**BSA**	**- 515**	**0**	**undetectable**

The fraction containing rhNGAL according to the ELISA results (E-[NGAL]-MSF+aa) was tested for NGAL bioactivity in the transmembrane assay using HSG and TYS as target cells. E-[NGAL]-MSF+aa did not affect the control or baseline migration when tested alone. However, in combination with rhMSF+aa (at 100pg/ml and 1ng/ml) it effectively inhibited MSF+aa-stimulated migration to baseline levels (Fig. 3.36 & 3.37). In contrast, it had no effect in combination with rhMSF-aa (Fig. 3.38).

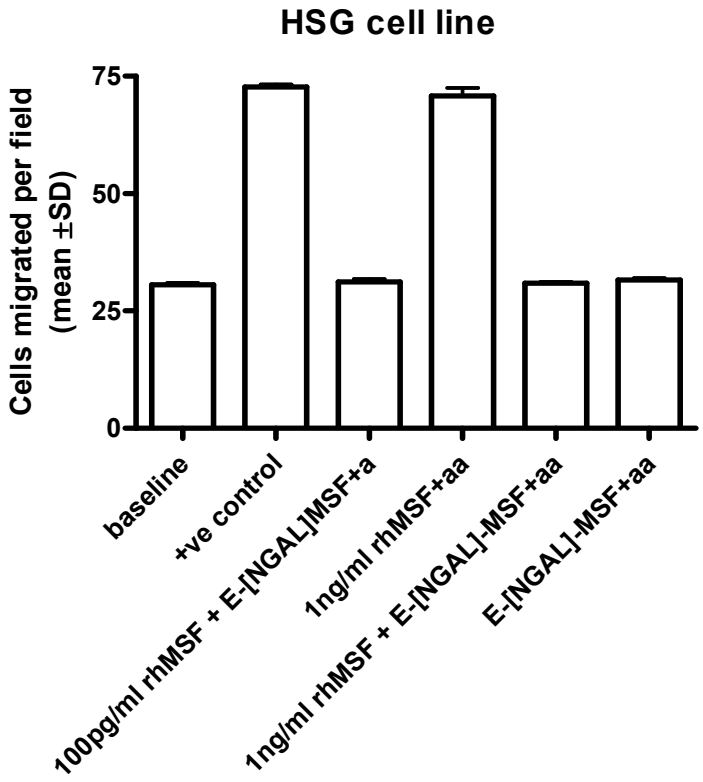


Figure 3.36: Effect of NGAL fraction eluted from MSF+aa column [E-(rhNGAL)-MSF+aa] on HSG target cells.

Motogenic activity was tested in the transmembrane assay using SF-MEM as a negative control (baseline) and 100pg/ml rhMSF+aa as a positive control. [E-(rhNGAL)-MSF+aa] did not affect baseline migration, but it inhibited the migration stmulated by MSF+aa (100pg/ml & 1ng/ml) to baseline levels. There was no significant effect of the eluted sample (NGAL) on the migration of the HSG cells when compared to the negative control (Bonferroni's test).

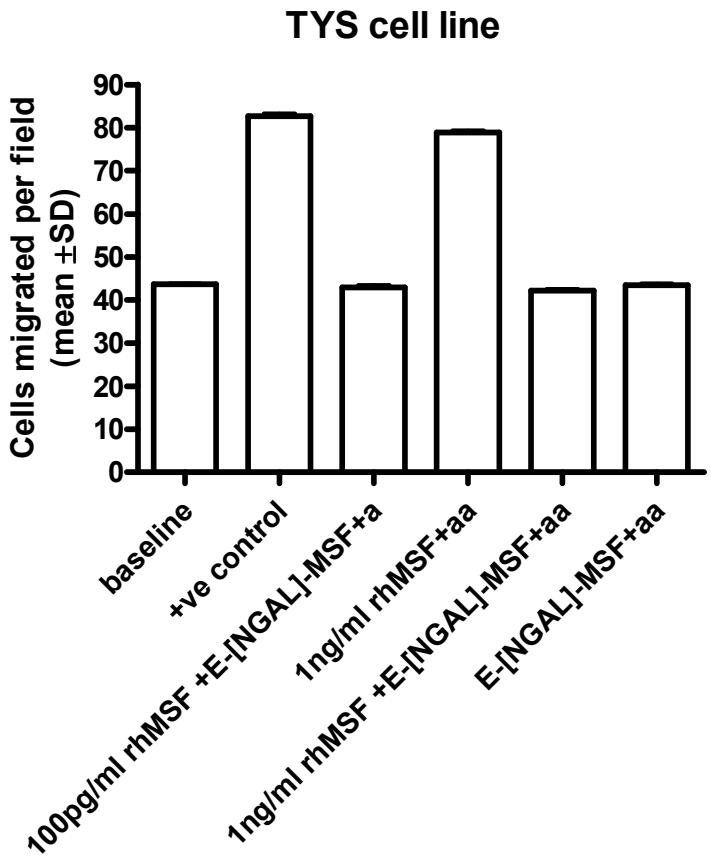


Figure 3.37: Effect of NGAL fraction eluted from MSF+aa column [E-(rhNGAL)-MSF+aa] on TYS target cells.

Motogenic activity was tested in the transmembrane assay using SF-MEM as a negative control (baseline) and 100pg/ml rhMSF+aa as a positive control. [E-(rhNGAL)-MSF+aa] did not affect baseline migration, but it inhibited the migration stmulated by MSF+aa (100pg/ml & 1ng/ml) to baseline levels. There was no significant effect of the eluted sample (NGAL) on the migration of the TYS cells when compared to the negative control (Bonferroni's test).

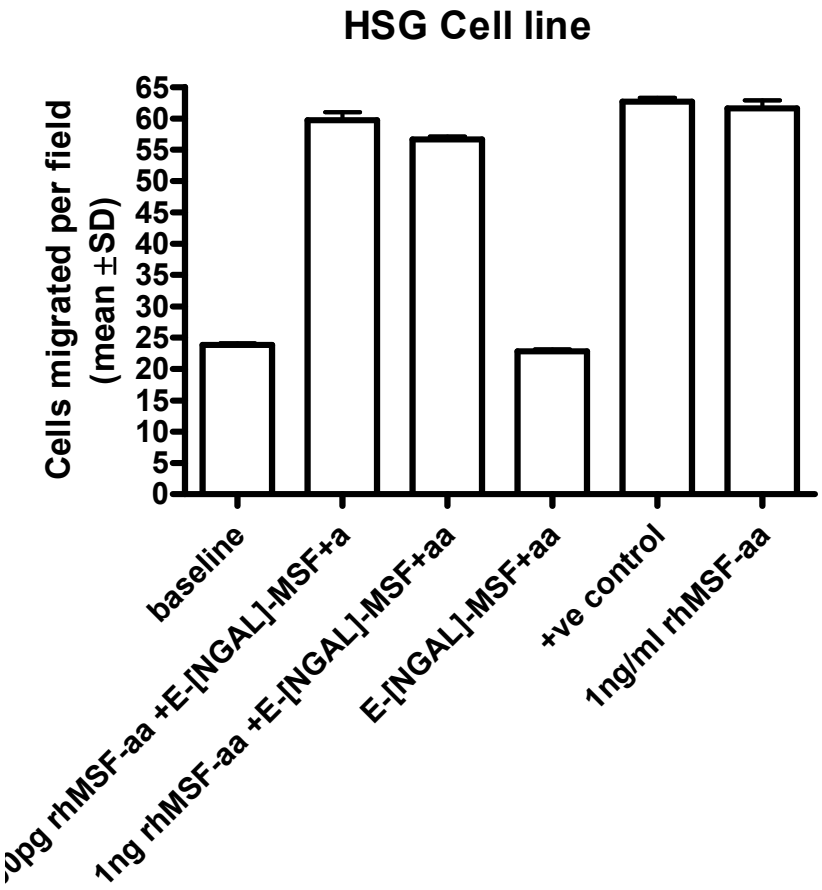
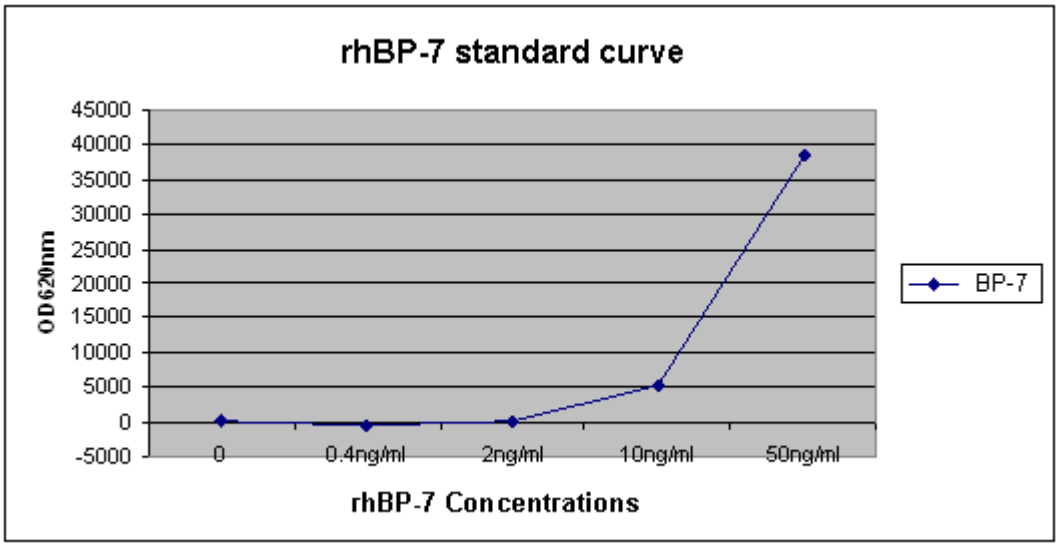


Figure 3.38: Effect of NGAL fraction eluted from MSF+aa column [E-(rhNGAL)-MSF+aa] on HSG target cells.

Motogenic activity was tested in the transmembrane assay using SF-MEM as a negative control (baseline) and 100pg/ml rhMSF-aa as a positive control. [E-(rhNGAL)-MSF+aa] did not affect baseline migration. There was no significant effect of the eluted NGAL on the migration of HSG cells when compared to the negative control (Bonferroni's test).

3.4.6.2 Binding of BP-7 to MSF

Following the same protocol as described for NGAL (3.4.6.1) I examined the possible binding of BP-7 to rhMSF+aa, rhMSF-aa and BSA by affinity chromatography. BP-7 was obtained from two different sources: (a) rhIGFBP-7 purchased from (R&D system) and (b) IGFBP7 purified from Endo742 CM by DEAE chromatography by Dr Sarah Jones This will be referred to as e-BP-7. (2µg/ml) rhBP-7 was incubated with the columns and eluted with 2M NaCl + 20mM Tris HCl pH 7.4. The resultant fractions were assayed for BP-7 concentration by indirect MSD ELISA using specific anti-BP-7 antibody (goat antibody AF 1334) (Fig. 3.39), as described in Materials and Methods Chapter two. Data presented in (Table 3.7) is representative of 3 separate experiments and indicate a direct association between BP-7 and both MSF isoforms. These results indicate a possible mechanism whereby BP-7 neutralises MSF bioactivity.



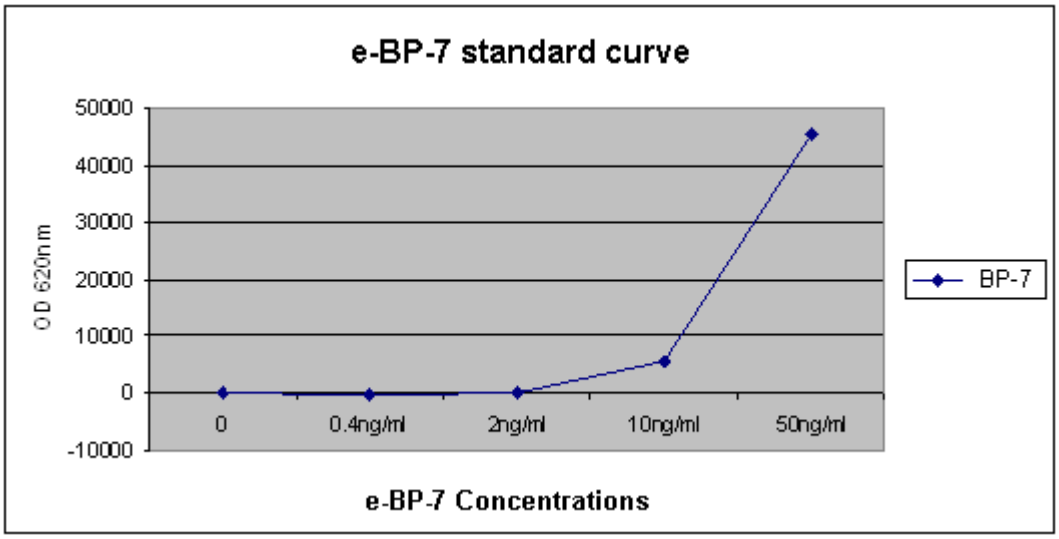


Figure 3.39: Indirect MSD ELISA standard curve of rhBP-7 and e-BP-7.

Table 3.7: MSD ELISA legend explains Ab and general methods.
Concentration of BP-7 in samples eluted from different affinity chromatography columns. The concentration was estimated by linear regression

Sample	Affinity column	OD (620nm) Blank subtracted		estimated concentration	Comments
		rhBP-7	e-BP-7		
E-[BP-7]-MSF-aa	MSF-aa	29820	112962	Less than 40ng/ml	detectable
E-[BP-7]-MSF+aa	MSF+aa	6761	103535.5	More than 10ng/ml	detectable
E-[BP-7]-BSA	BSA	-187.5	-200	0	undetectable

The transmembrane assay on TYS cells was used to identify BP-7 bioactivity in the fractions eluted from MSF+aa affinity column chromatography [E-(rhBP-7)-MSF+aa] and [E-(e-BP-7)-MSF+aa].These fractions did not affect the control or baseline migration when tested on their own, but in combination with rhMSF+aa at 100pg/ml and 1ng/ml it effectively inhibited cell (TYS) migration to baseline levels (Fig 3.40 & 3.41).

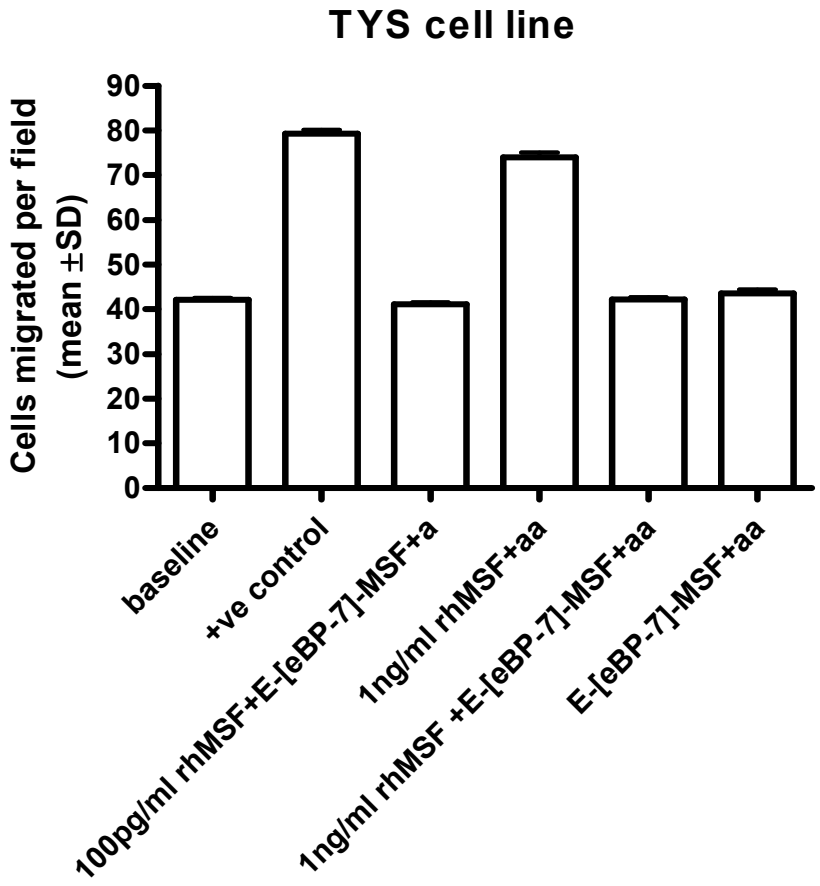


Figure 3.40: Effect of BP-7 fraction eluted from MSF+aa column [E-(e-BP-7)-MSF+aa] on TYS target cells.

Motogenic activity was tested in the transmembrane assay using SF-MEM as a negative control (baseline) and 100pg/ml rhMSF-aa as a positive control. Addition of [E-(e-BP-7)-MSF+aa] recovered in the material bound to MSF+aa affinity column reduced the migration of TYS cells to baseline levels. There was no significant effect of [E-(e-BP7)-MSF+aa] on the migration of TYS cells when compared to the negative control (Bonferroni's test).

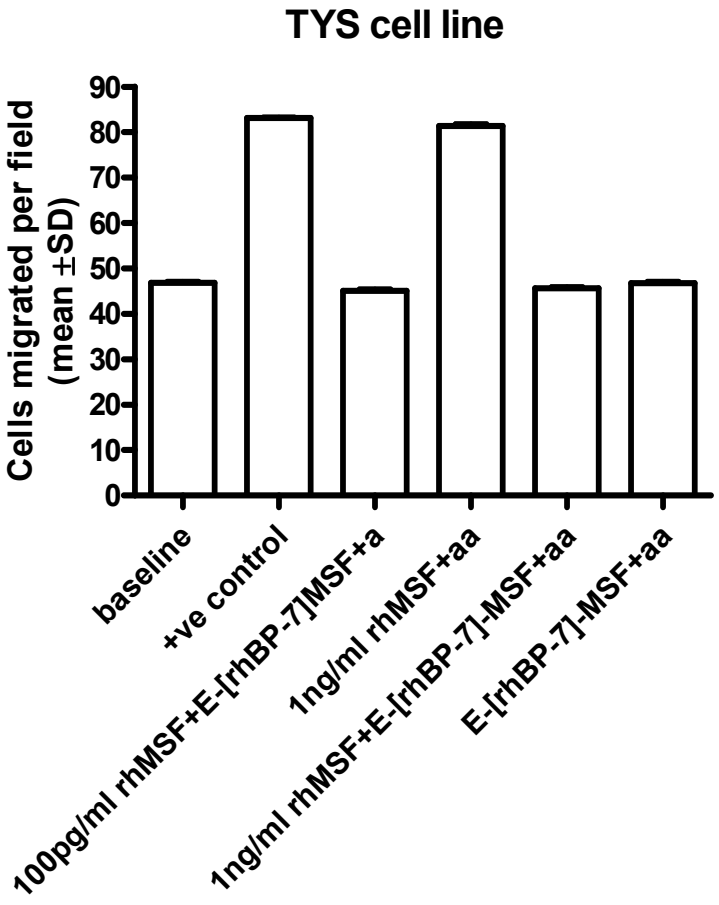


Figure 3.41: Effect of BP-7 fraction eluted from MSF+aa column [E-(rhBP-7)-MSF+aa] on TYS target cells..

Motogenic activity was tested in the transmembrane assay using SF-MEM as a negative control (baseline) and 100pg/ml rhMSF-aa as a positive control. Addition of [E-(rhBP-7)-MSF+aa] recovered in the material bound to MSF+aa affinity column reduced the migration of TYS cells to baseline levels. There was no significant effect of [E-(rhBP7)-MSF+aa] on the migration of TYS cells when compared to the negative control (Bonferroni's test).

3.5 Discussion

MSF is a potent motogenic factor, able to stimulate the migration of fibroblasts, epithelial and endothelial cells. MSF also stimulates HA synthesis and angiogenesis (Schor *et al*., 1988 and Grey et al., 1989). Cell migration is most commonly studied using the transmembrane or Boyden Chamber assay. By using this assay, the chemotactic activity of rhMSF and its mutants have been examined on different human cell types, namely TYS (oral tumour cell line) HSG (salivary tumour cell line) Endo 742 (microvascular endothelial cells) and FSF44 (foreskin fibroblast).

The aims of this study were to determine the expression of MSF by oral tumour cell lines, the effects of MSF on the different types of cells, the amino acid motifs which are responsible for MSF bioactivity and the modulation of MSF bioactivity by antibodies and other proteins. In this study previous results are confirmed and novel data is presented, as discussed under the following headings:

3.5.1 Identification of MSF isoforms

Two MSF isoforms have been cloned. These differ by a 15 amino acid deletion in the first type II fibronectin structural module and are referred to as MSF+aa and MSF-aa (see chapter one, Introduction). The general term MSF (or total MSF) will be used to refer to both isoforms. In this thesis I demonstrate that

(a) VSI antibodies (to C-terminus decapeptide VSIPPRNLGY) recognise both isofrms MSF+aa and MSF-aa.

(b) TYN antibodies (TYNDRTDSTTSNY, an amino acid sequence that is present in MSF-aa only) recognise MSF-aa but not and MSF+aa.

(c) Both isoforms of MSF are produced by TYS and HSG cell lines

(d) MSF+aa and MSF-aa differ in their interaction with NGAL

(e) The bioactive motifs of MSF are different for fibroblasts, endothelial cells and oral or salivary tumour cells

3.5.2 Production of MSF by tumour cell lines

Conditioned medium from oral tumour cell line (TYS) was collected. This was tested for migration stimulating activity, which gave a positive result. The CM was then tested for MSF by affinity chromatography to investigate if a soluble factor secreted into the medium stimulates migration. Testing of the eluted fractions on their

own or by both RpVSI and TYN affinity columns revealed the presence of MSF and the bioactivity of this conditioned medium in promoting the migration of oral tumour cell lines and other cell types was ascertained using the transmembrane migration assay. The MSF found was further characterised by its sensitivity to the MSF inhibitors NGAL and both human recombinant rhBP-7 and endothelial purified (e-BP-7) and to the functional neutralising antibodies PEPQ. The results indicated that baseline migration showed more than two fold increases in cell migration with CM alone or in the eluted fractions from RpVSI and TYN affinity columns, also with different concentrations of rhMSF+aa, but in combination with MSF inhibitors and functional neutralising antibodies PEPQ effectively inhibited the cells migrated (HSG, TYS, Endo and FSF44) from values comparable to those of positive controls to baseline levels. These result indicated that conditioned medium from TYS contained both types of MSF isoforms, which is confirmed by the inhibitory effect of rhNGAL to the MSF+aa rather than to MSF-aa, on different target cells.

The presence of MSF in the bioactive CM of TYS cells was confirmed by the specific abrogation of motogenic activity by the MSF-specific function-neutralising antibody and by affinity chromatography with MSF-specific identification antibodies.

3.5.3 Effects of MSF on different target cells

The motogenic activity of MSF was investigated, because cell migration is an important feature of tumour progression. Our results indicated that rhMSF+aa and rhMSF-aa stimulate the migration of different cell types oral tumour cell lines (TYS), salivary tumour cell line (HSG), endothelial cells (Endo 742), and foreskin fibroblast cell line (FSF44) ($p<0.05$). The profile of cell migration at different concentrations produced a bell shaped dose-response curve in TYS, Endo 742 and FSF44 demonstrating that for initiation of cell migration a threshold concentration is required and at higher concentrations a negative feed back mechanism occurs. This result is similar to the previous observations (Ellis *et al.,* 2010). On the other hand, in the case of HSG cells all concentrations of MSF tested (1pg/ml-1µg/ml)) exhibited significant motogenic activity by comparison to the negative control or baseline, with a plateau reached at 10 ng/ml. Schor *et al.,* (1999) have previously shown that IGD motif stimulated fibroblast cell migration and it depends on both integrin αvβ3 functionality and tyrosine phosphorylation of FAK. Integrin clustering and/or downstream signalling may be the possible reasons of reduced migration at higher concentrations.

3.5.4 Bioactive motifs of MSF

In order to investigate the possible active amino acid motif for these cells migration stimulation, rhMSF +aa mutants have been used as followed; MSF+3,5m (No IGD 3, 5); MSF+7,9m (No IGD7, 9); MSF QUAD mutant (No IGD motif in its structure i.e. all the four IGD motif has been mutated to DGI) and MSF+HEEGH mutant (No FEEGF motif) and MSF+QmHm (No IGD and FEEGF). Mutation of the IGD motifs separately or all together inhibited the migration stimulating activity of MSF to the baseline level in both TYS and HSG cell lines. Mutation of MSF HEEGH motif and mutation of both the IGD and HEEGH motif also show similar results to the MSF QUAD mutant, which provide evidence that both IGD and HEEGH motifs are required for the stimulation of migration of the oral tumour cell lines (TYS) and salivary gland tumour cell lines (HSG). In contrast, mutation of IGD either separately or all together, and mutation of HEEGH motif alone had no effect on the migration stimulating activity of MSF on the endothelial cells (Endo 742). Whilst mutation of both MSF QUAD and HEEGH motifs together inhibited the migration of endothelial cells to the baseline level, this indicated that both the HEEGH and IGD motifs in MSF must be mutated in order to abolish the motogenic response of these cells.

MSF also may affect cell migration through its fibronectin proteinase activity by directly digesting ECM molecules or by processing cell surface molecules. Houard *et al,* (2005) shown that Fn-proteinase activity was totally abolished when 2 histidine residue from HEEGH motif were replaced by phenylalanine (FEEGF). This type of mutation is known to eliminate the catalytic activity of zinc-dependent metalloenzymes (Wang and Cooper, 1993). Houard *et al,* (2005) also suggested that MSF migratory activity was dependent on Fn-proteinase activity when they observed 65% inhibition of breast adenocarcinoma cells (MCF-7) migration after mutating the HEEGH motif. Our data presented here indicated that five bioactive motifs (four IGD and HEEGH) are required for migration stimulating activity of MSF.

3.5.5 Modulation of MSF bioactivity by antibodies and other proteins

Data presented in this study indicate that both MSF isoforms display the same spectrum of potent motogenic bioactivities and these are effectively inhibited by PEPQ function-neutralising antibodies, this is in agreement with the result (regarding MSF+aa) of Schor *et al.,* (2003) on fibroblast cells. MSF+aa and MSF-aa differ in their functional interaction with NGAL (neutrophil gelatinase-associated lipocalin) an inhibitor of MSF present in serum and certain tumours (Jones *et al.,* 2007): MSF+aa is inhibited by NGAL, whereas MSF-aa is not. Both isoforms contain the same MSF-specific 10 amino sequence and IGD functional domains; consequently they are equally active in the absence of NGAL. In addition to that our results suggest a mechanism of action by a direct association between NGAL and MSF+aa, whilst there is no binding with MSF-aa. However, the direct association between NGAL and MSF+aa, reflect that the possible mechanism of action whereby MSF+aa is inhibited by NGAL, whereas MSF-aa is not. In contrast, Jones *et al,* (2007) reported that no evident of binding between MSF and NGAL in the keratinocyte conditioned media, they suggested that NGAL may block an MSF activated signal transduction pathway or may be active indirectly by inducing the expression of an intermediate inhibitory factor.

This is the first report of the inhibitory effect of BP-7 on both MSF isoforms motogenic activities. In addition to that, these data indicate a direct association between BP-7 and both MSF isoforms, which may reflect the mechanism of action whereby BP-7 neutralises MSF bioactivity. Our data indicate a novel function of BP-7 and its possible role in inhibition of MSF bioactivity.

3.6 Conclusions

- Tumour cell lines derived from salivary gland tumours or from oral tumours secreted bioactive MSF in culture. MSF stimulated the migration of these tumour cells. *In vitro* mutagenesis indicated that five bioactive motifs are required for MSF bioactivity.

3.7 Further studies

- Using other types of tissue target cells.
- Investigate the role of other soluble molecules on the MSF bioactivity.

4. Chapter four: MSF Expression in Oral squamous cell carcinoma

4.1. Introduction

Oral cancer is the most common type of cancer in developing countries, where it constitutes a major health problem and commonly leads to death. More than 750,000 patients worldwide suffer from oral cancer (Parkin *et al.,* 2005).

In the United Kingdom there are approximately 5400 new cases of oral cancer each year and approximately 1800 deaths (Cancer Research UK, 2007). Scotland has the highest occurrence of oral cancers in the UK; the incidence is still increasing. Survival rates of oral cancer are still very poor with 42% for men and 48% for women five years survival reported, and these have not improved in the last few decades (Cancer Research UK, 2007) despite advances in the therapeutic interventions and our increased understanding of the disease. The results of standard therapy, including surgery and/or irradiation, have remained at a disappointingly stable level for many years despite significant development in the multimodal treatment of the disease. Furthermore, the current cornerstones of therapeutic decision-making, namely the tumour-node-metastasis (TNM) system supplemented with conventional histopathological tumour grading, have proven to be imperfect prognostic indicators. There is therefore a continuing need to evaluate potential new biomarkers that may be useful in diagnosis and prognosis. The most common type of oral cancer is squamous cell carcinoma. For further background information see Chapter one.

4.2. Aims

The aims of this study were to determine (i) the expression of total MSF and MSF-aa in oral squamous cell carcinoma (OSCC) and adjacent non-tumour tissues; and (ii) evaluate the potential usefulness of MSF expression in the diagnosis and prognosis of OSCC.

4.3. Materials and Methods

4.3.1 Specimens: OSCC study

A total of 64 paraffin-embedded archival oral SCC specimens were obtained from the Domagk-Institute of Pathology, University Clinic of Munster, Germany. The available details of the patients are presented in Table 4.1. Adjacent non-tumour tissue, (also stained and evaluated in this study), included regions of histologically

normal salivary gland (NSG, in 31/64 specimens) and epithelial hyperplasia (Hp, in 37/64specimens).

4.3.2 Immunohistochemistry:

Standard immunohistochemical procedures were used to stain paraffin embedded OSCC with two antibodies: mab7.1 (Schor *et al.,* 2003) and mabTYN 1.2, as outlined in chapter two (Materials and Methods). Mab7.1 recognises MSF+aa and MSF-aa (total MSF) whereas mabTYN 1.2 only recognises MSF-aa (Chapter three).

4.3.3 Assessment of MSF expression

Expression of MSF was assessed as described in Chapter two (Materials and Methods). MSF overall grade was determined separately in areas of the invasive tumour front (ITF) and in the keratin pearl areas in the centre of the tumours (Kp), as negative (grade 0), weak (grade 1), moderate (grade 2), or strong (grade 3) positive. The % of area stained, final score, stroma and adjacent non-tumour tissues were assessed in the whole section.

4.3.4 Statistical analyses

The two-tailed Mann–Whitney tests were used to determine the difference in MSF isoform expression. Correlation between MSF at the ITF and other clinicopathologic parameters were studied by Fisher Exact and chi-squared tests. Survival probabilities were estimated by the Kaplan-Meier method and survival curves were compared with the log-rank test. *P* value lesser than or equal to 0.05 was considered as statistically significant.

Table 4.1: Summary of clinical details of OSCC patients

Parameters	Classification	No of patients for Ab 7.1	No of patients for Ab TYN
Pathological grade	Grade 1	7	6
	Grade 2	51	40
	Grade 3	6	4
	All	N=64	N=50
Sex	1= Male	47	42
	2=Female	8	8
	All	N=55	N=50
Age (years)	Range Mean Median	33-80 53.9 54	33-80 54.5 54
	All	N=55	N=50
Censor	0 =Alive	22	20
	1 =Dead	33	30
	All	N=55	N=50

4.4. Results

4.4.1 Immunolocalisation of total MSF and MSF-aa in oral squamous cell carcinoma (OSCC) and adjacent non-tumour tissue

Duplicate sections of OSCC were stained with antibodies mab7.1 (for total MSF, referred to also as MSF) and TYN (for MSF-aa). Both antibodies stained most of the tumours examined, with heterogeneous staining observed in the carcinoma and associated stromal tissue, including fibroblasts, blood vessels and inflammatory cells. Histologically normal salivary gland (NSG) and epithelial hyperplasia (Hp) were present adjacent to some of the tumours. Representative examples of total MSF and MSF-aa expression by OSCC and associated stromal cells are presented in (Fig 4.1, 4.2D and Fig 4.3A, B, C, F). Staining in the carcinoma cells was generally stronger in the keratin pearl central areas (Kp) than in the invasive tumour front (ITF). Positively stained acinar cells were rarely present in NSG, whilst most of salivary ducts were positively stained (Fig 4.2C and Fig 4.3E). In the adjacent epithelial hyperplasia, positively stained suprabasal cells (Hpsb) were detected in some sections, whilst no staining was found in the basal cell layers (Hpb) (Fig 4.2A and Fig 4.3D). Sections incubated with normal mouse IgG, instead of MSF antibody (negative controls), showed no staining (Fig 4.2E and Fig 4.3G).

4.4.2 Differences in total MSF expression among tissues

MSF expression was first graded as negative (0), weak positive (1), moderate (2) or strong positive (3) by three independent observers. Keratin pearl (Kp) and invasive tumour front (ITF) areas were evaluated separately. The semi-quantitative results obtained are summarised in Table 4.2. Results presented in Fig 4.4A and 4.4B demonstrate a significant difference in MSF overall grade between Kp and ITF, indicating that overall MSF expression was significantly higher in Kp than in ITF ($P<0.0001$, 0.0029).

The overall MSF grade gives a general assessment of the staining in OSCC tissue sections, including epithelial and stromal compartments. More detailed observations of the tumour-associated stroma revealed that MSF was heterogeneously distributed among stromal cells, fibroblasts, blood vessels and inflammatory cells, with some amount of staining being detected in most specimens. The percentage of specimens that showed positive fibroblasts, blood vessels and inflammatory cells represented 94%, 94% and 78% of the total, respectively (Fig 4.5A).

MSF was also detected in certain non-tumour structures adjacent to OSCC (Fig 4.6A). Overall MSF grade of the NSG reflected mainly the staining present in the acinar cells. More detailed observation of the different NSG cell types indicated that mucous and serous acinar cells were indeed predominantly negative for MSF, whereas ductal cells commonly exhibited a diffuse positivity, this being stronger in the luminal cells compared with basal and myoepithelial cells. Of 31 NSG specimens examined, 18, 23 and 28 (58%, 74% and 90%) showed positive intercalated, striated and excretory salivary ducts, respectively. Only 3 specimen (5%) showed MSF staining in acinar (serous) cells and 16 (52%) in myoepithelial cells. Further information on NSG is presented in chapter five.

In the epithelial hyperplasia, staining was restricted to the suprabasal cell layers (Hpsb) (Fig 4.6A). The percentage of specimens that showed positive staining was similar when comparing Hpsb, Kp and ITF assessments; all these three being significantly higher than for Hpb or NSG ($P< 0.0002–0.0001$).

Table 4.2: Tissues compared and P value (for number and %) for Ab 7.1

Tissues compared	MSF grades compared (Ab 7.1)			
	for actual number of specimens (n)			for % of specimens
	0/1 v 2/3 (a)	0 v 1/2/3 (a)	0 v 1 v 2/3 (b)	0/1 v 2/3 (a)
KP v ITF	<0.0001	<0.0029	< 0.0001	< 0.0001
KP v NSG	<0.0001	<0.0001	< 0.0001	< 0.0001
ITF v NSG	<0.0024	<0.0001	< 0.0001	< 0.0001
KP v Hpsb	<0.0001	<0.366	< 0.0001	< 0.0001
ITF v Hpsb	<0.0959	<0.0877	<0.0785	<0.0155
Hpsb v NSG	<0.0001	<0.0001	< 0.0001	< 0.0001
KP v Hpb	<0.0001	<0.0001	< 0.0001	< 0.0001
ITF v Hpb	<0.0001	<0.0001	< 0.0001	< 0.0001
Hpb v NSG	<0.204	<0.089	< 0.153	< 0.028
Hpb v Hpsb	<0.0001	<0.0001	< 0.0001	< 0.0001

(a)Fisher's exact test; (b) Chi-squared test, Hpsb= hyperplasia in suprabasal layers, Hpb=hyperplasia in basal layer, NSG= normal salivary gland

The percentage of MSF-positive specimens may be represented as NSG=Hpb< Hpsb=ITF<K.

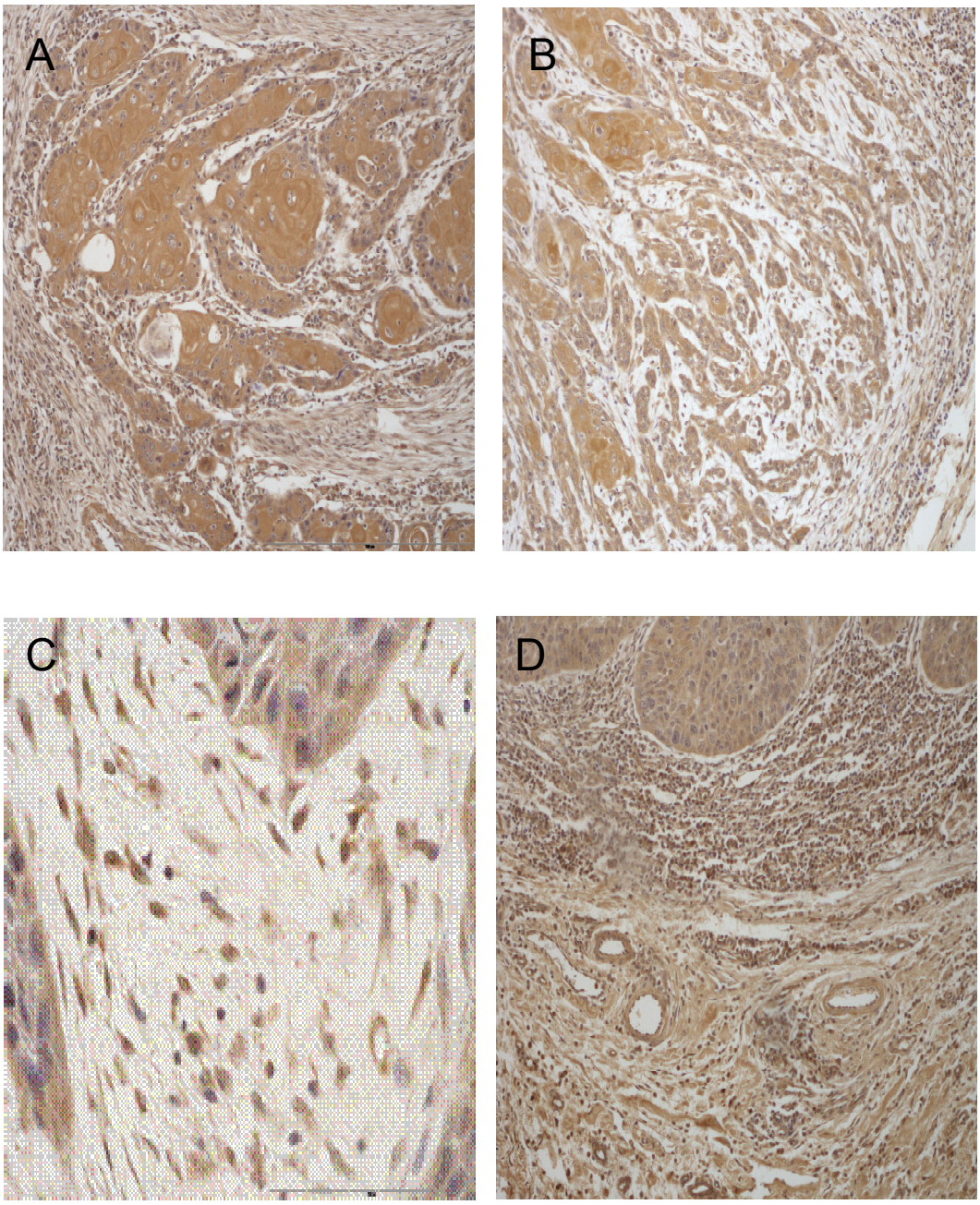


Figure 4.1: Immunolocalisation of total MSF in OSCC.
(A, B) Total MAF expression in Kp (A) and ITF (B). (C, D) showing total MSF staining in the tumour cells, associated blood vessels and fibroblasts. Original photographs were taken at magnification x100 (A, B, D) or x 400 (C).

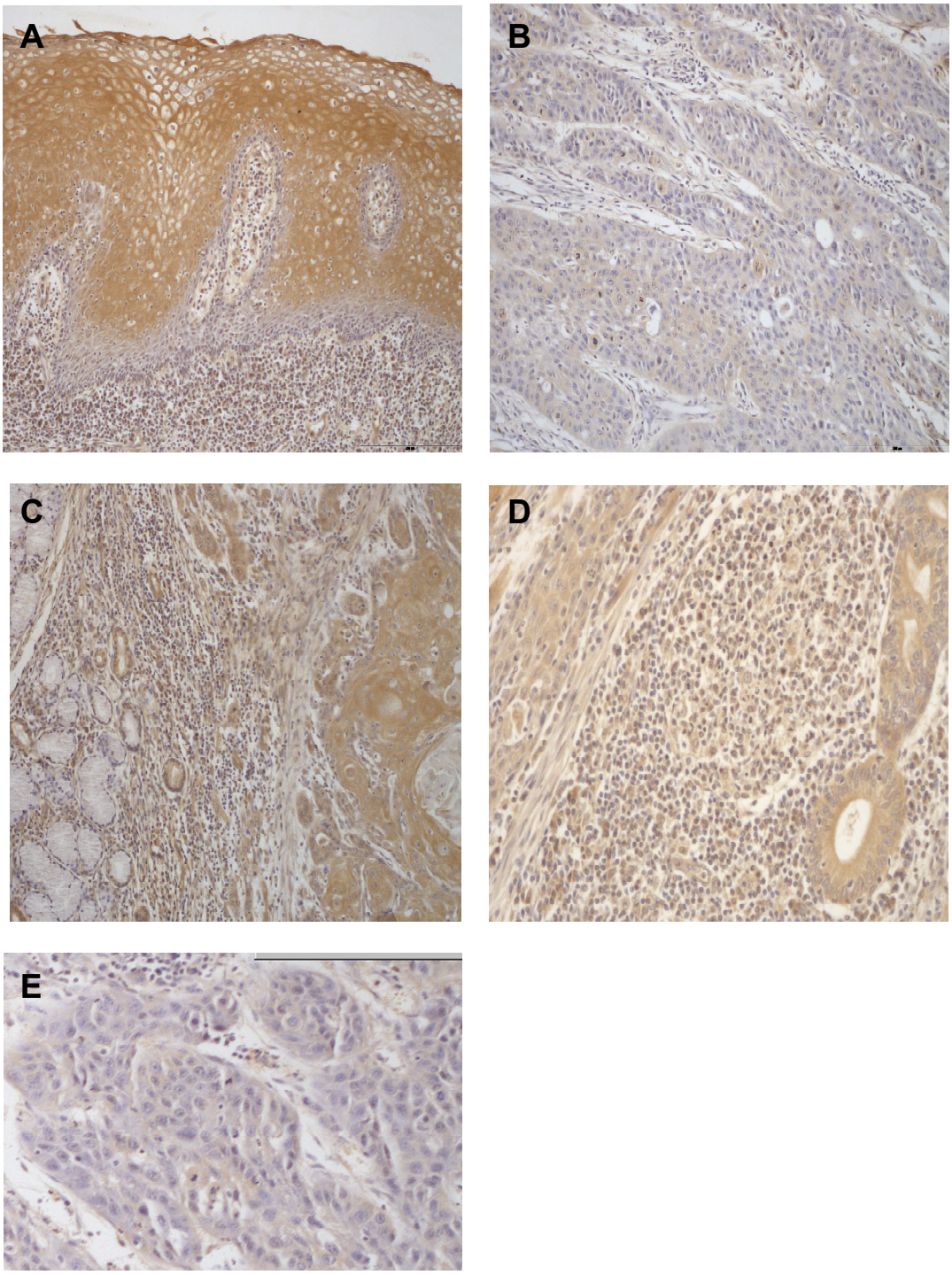
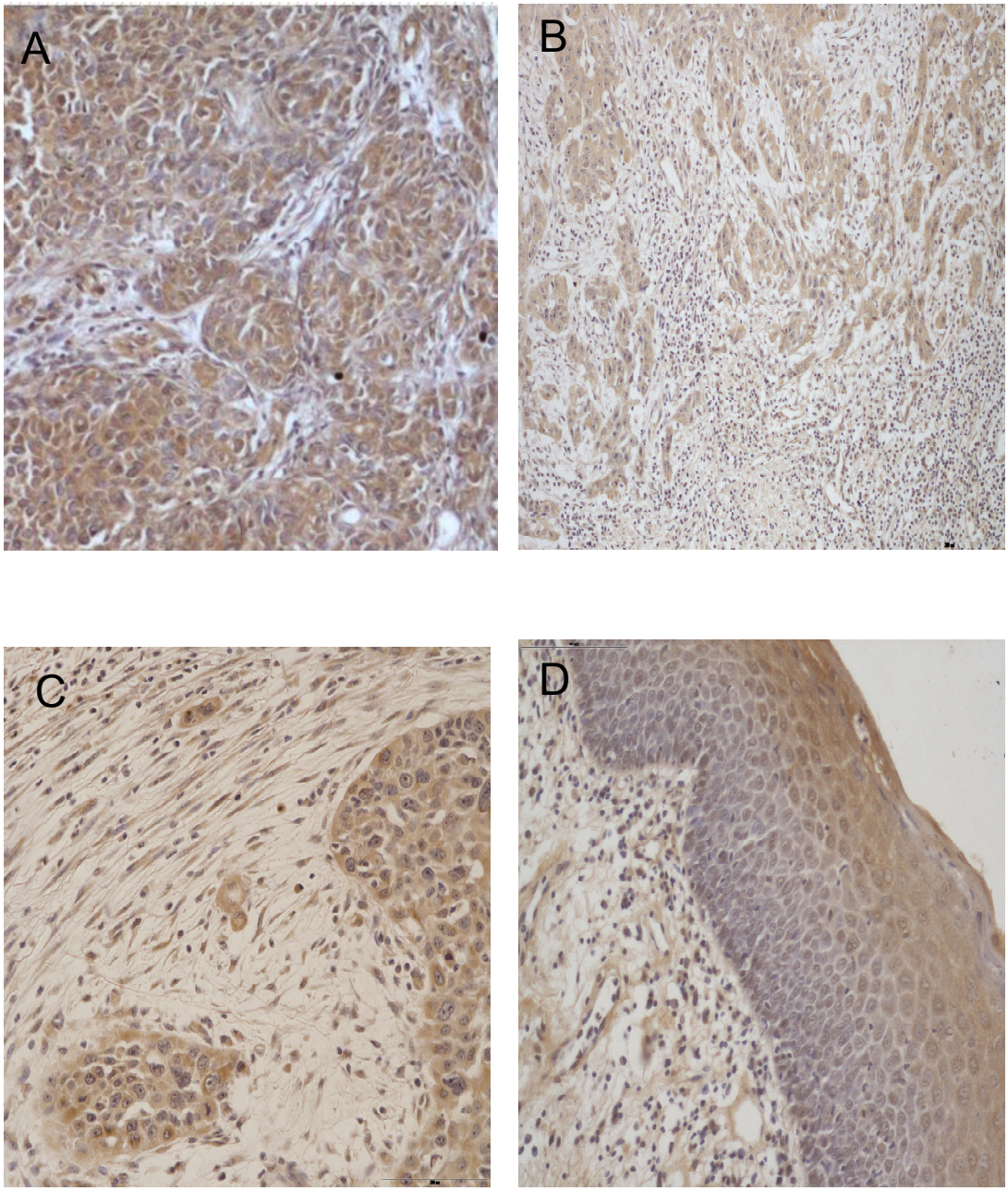


Figure 4.2: Immunolocalisation of total MSF in OSCC.
(A) Positive total MSF staining in the Hpsb and negative staining in Hpb. (B) Negative total MSF staining in OSCC. (C) Negative total MSF staining in histologically normal salivary gland (NSG) (mucous cells) adjacent to positive OSCC. (D) Positive inflammatory cells and carcinoma cells. (E) Showed no staining with normal mouse IgG, instead of MSF antibody (negative control). Original photographs were taken at magnification x 100.


A
B
C
D

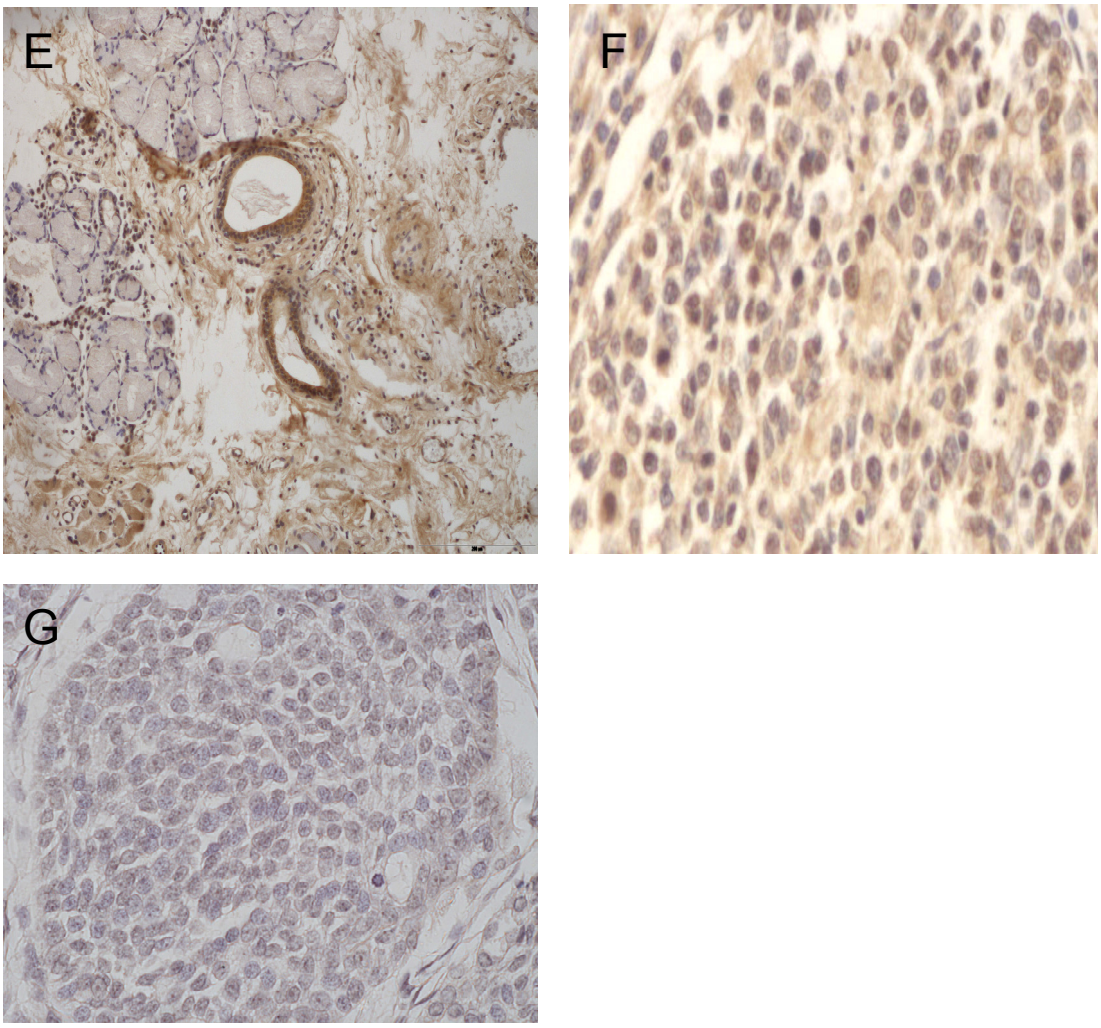


Figure 4.3: Immunolocalisation of MSF-aa in OSCC.
(A, B) MSF-aa expression in Kp (A) and ITF (B). (C) Showing MSF-aa staining in the tumour cells and associated blood vessels and fibroblasts. (D) Positive MSF-aa staining in the Hpsb and negative staining in Hpb. (E) Negative MSF-aa staining in histologically normal salivary gland (NSG) (mucous cells) and positive ductal epithelial cells adjacent to positive OSCC. (F) Positive inflammatory cells. (G) Showed no staining with normal mouse IgG, instead of MSF antibody (negative control). Original photographs were taken at magnification x 100 (A, B, D, E, G) or x 400 (C, F).

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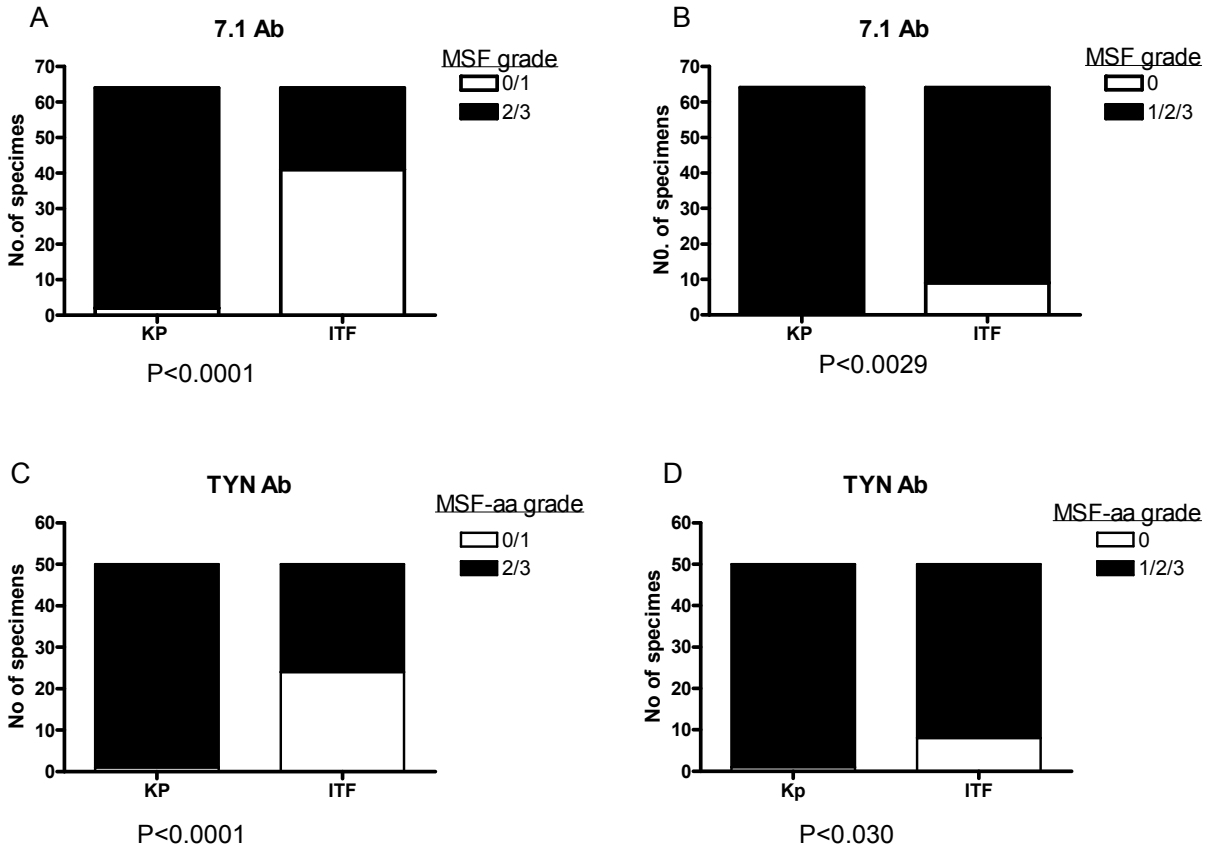


Figure 4.4: MSF overall grade in the OSCC.
The tissues were stained with 7.1 and TYN antibodies, as indicated. MSF expression in the epithelium of keratin pearl (Kp) (n= 64, 50) and invasive tumour front (ITF) (n=64, 50) for 7.1 Ab & TYN Ab respectively was evaluated and compared. Significant differences between Kp and ITF occurred in terms of the MSF overall grade. (Fisher's Exact Test).

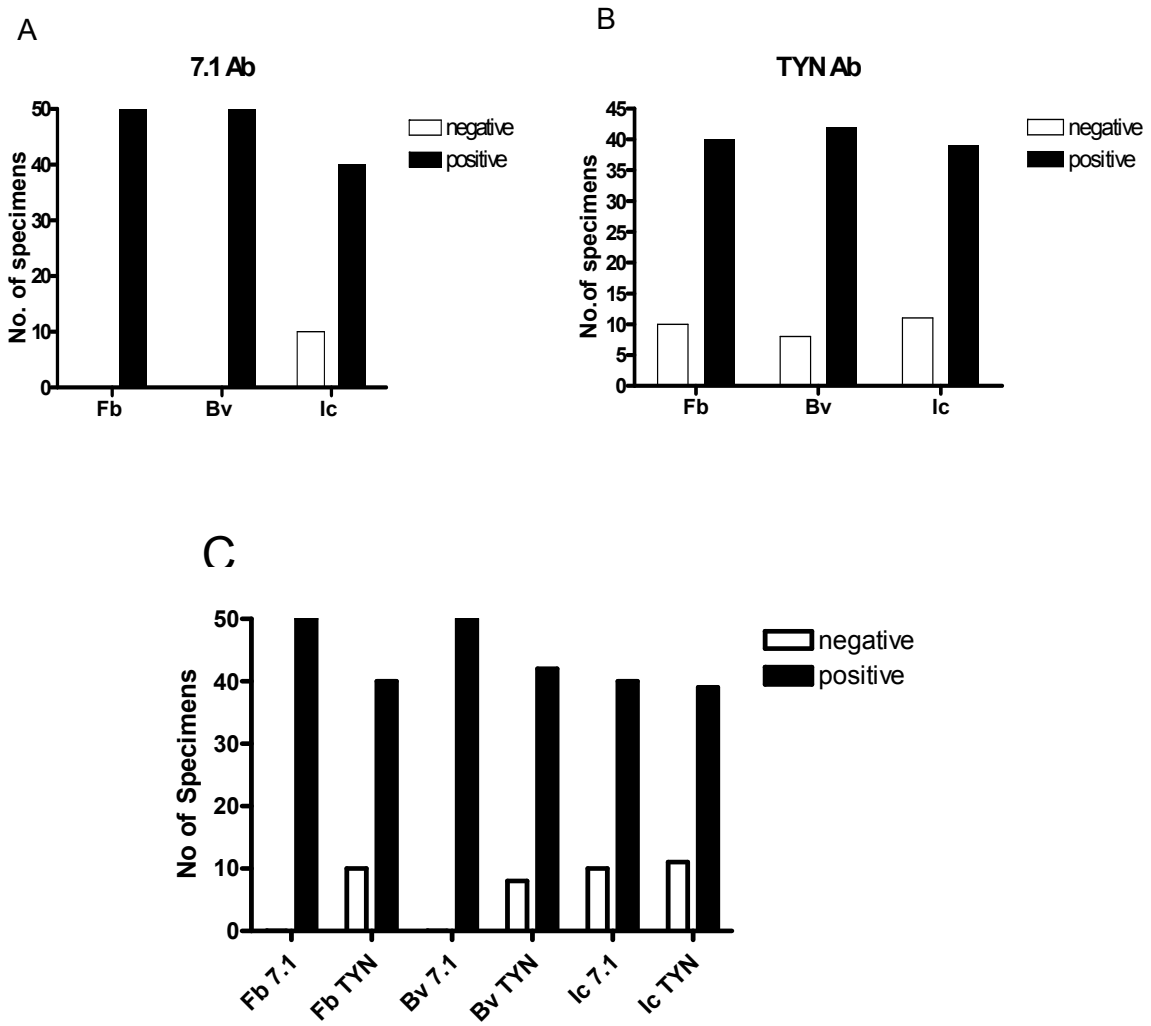


Figure 4.5: Stromal MSF (Both isoforms) expression in the OSCC.
Results show the percentage of specimens graded positively stained by antibody 7.1 (A) and TYN (B) for different cellular compartments, including fibroblasts (Fb); blood vessels (Bv) and inflammatory cells (Ic). The difference between the two antibodies (C) was analysed by Fisher's exact test for the actual number of specimens assessed (n) and for the percentage of specimens (%, as shown in the graph). The respective p values for (n) and (%) were: 0.001 and 0.0002 for Fb; 0.005 and 0.001 for Bv; and there was no significant difference between the two antibodies regarding Ic.

4.4.3 Differences in MSF-aa expression among tissues

Specimens stained with mabTYN (for MSF-aa) were graded according to MSF overall grade, as for mab7.1. The assessment included 50 OSCC specimens. Adjacent NSG was present in 26/50 and Hp in 31/50 of these specimens.

The results are summarised in Table 4.3. MSF-aa was detected in Kp in 98% (49/50) specimens and in ITF in 84% (42/50) specimens (Fig 4.4C, D). In the adjacent tissue, MSF-aa was found in the NSG in 8% cases (2/26) (serous cells), in Hpsb in 97% cases (30/31) and in Hpb in 0% cases (0/31). In the stromal compartment of OSCC (Fig 4.5B), positive stained specimens represented 80%, 84% and 78% of total specimens examined for fibroblasts, blood vessels and inflammatory cells, respectively.

Table 4.3: Tissues compared and P value (for number and %) for Ab TYN

Tissues compared	MSF grades compared			
N= 50	for actual number of specimens(n)			for % of specimens
	0/1 v 2/3 (a)	0 v 1/2/3 (a)	0 v 1 v 2/3 (b)	0/1 v 2/3 (a)
KP v ITF	<0.0001	0.030	< 0.0001	< 0.0001
KP v NSG	< 0.0001	< 0.0001	< 0.0001	< 0.0001
ITF v NSG	< 0.0001	< 0.0001	< 0.0001	< 0.0001
KP v Hpsb	<0.0001	Ns 1	0.0001	< 0.0001
ITF v Hpsb	<0.040	0.142	0.0053	<0.0003
Hpsb v NSG	0.0172	<0.0001	< 0.0001	< 0.0001
KP v Hpb	<0.0001	<0.0001	< 0.0001	< 0.0001
ITF v Hpb	<0.0001	<0.0001	< 0.0001	< 0.0001
Hpb v NSG	<0.456	<0.203	0.290	< 0.121
Hpb v Hpsb	0.002	<0.0001	< 0.0001	< 0.0001

(a)Fisher's exact test; (b) Chi-square test, Hpsb= hyperplasia in suprabasal layers, Hpb=hyperplasia in basal layer, NSG= normal salivary gland

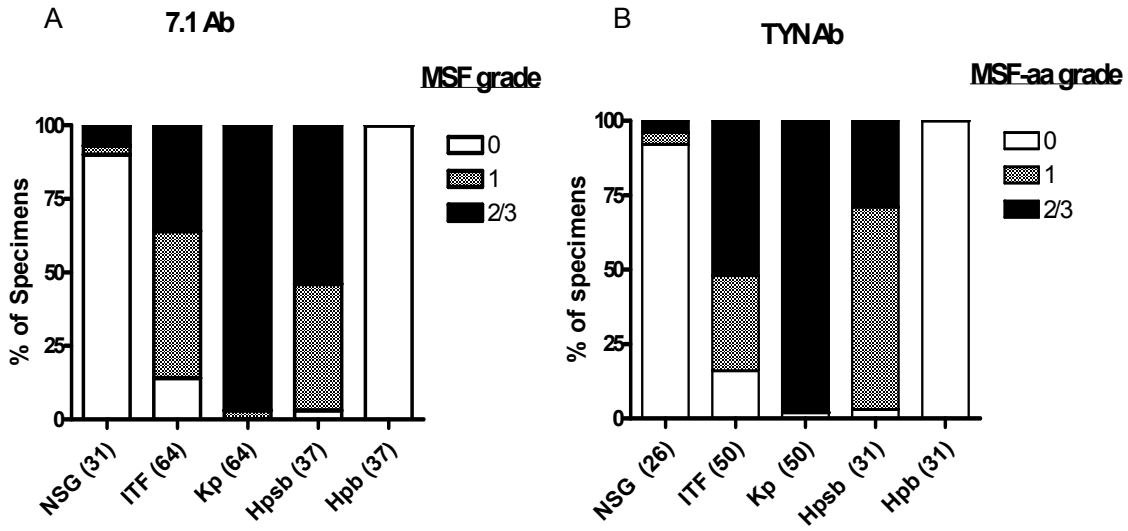


Figure 4.6: Percentage of OSCC specimens showing the indicated MSF (Both isoforms) grades
The tissues were stained with antibody 7.1(A) and TYN (B), as indicated. The tissues examined included histologically normal salivary gland (NSG, n=31or 26), pathological invasive tumour front (ITF) (n=64, 50), keratinpearl (Kp) (n=64, 50), hyperplasia suprabasal cell layers (Hpsb) (n=37, 31) and hyperplasia basal cell layers (Hpb)(n= 37, 31). Chi square tests (MSF grades 0 v. 1 v. 2/3) and Fisher's exact Test (MSF grades 0 v. 1/2/3) demonstrated statistical tests as shown in (Table 4.2 & 4.3).

4.4.4 Comparison of staining with antibodies 7.1 and TYN:

Fifty specimens were stained with both anti total MSF antibody (7.1) and anti-MSF-aa antibody (TYN). Comparison of the staining in these specimens is shown in Table 4.4 and Fig 4.7. Most OSCC specimens stained positively with both antibodies; however, a small number (1/50) showed positive staining with 7.1 but were negative with TYN. No significant differences were found between the two antibodies regarding MSF overall grades in Kp (P=1) and in ITF (P<0.553) (Fig 4.7). However, significant differences (7.1 > TYN) were found regarding percentage of area stained and final score (P< 0.008 and P<0.016) respectively (Fig 4.8), significant differences (7.1 > TYN) were also observed regarding MSF overall grade in stromal

fibroblasts (P< 0.001) and blood vessels (P< 0.005), but not in the inflammatory cells (Fig 4.5C).

No significant differences were observed in the NSG for both antibodies (Fig 4.7), acinar cells were predominantly negative whereas ductal cells commonly exhibited a diffuse positivity, this being stronger in the luminal cells compared with basal and myoepithelial cells. However, there was no significant difference between the two antibodies regarding epithelial hyperplasia**.**

Table 4.4: Statistical comarison of antibodies; Tissues and p value (for number and %) between Abs 7.1 and TYN

		MSF grades compared			
Tissues compared	**Abs**	**for actual number of specimens(n)**			**for % of specimens**
		0/1 v 2/3 (a)	**0 v 1/2/3 (a)**	**0 v 1 v 2/3 (b)**	**0/1 v 2/3 (a)**
KP v KP	**7.1 &TYN (n=50)**	**1**	**1**	**0.367**	**0.497**
ITF v ITF	**7.1 &TYN (n=50)**	**0.105**	**0.553**	**0.053**	**0.015**
Hpsb v Hpsb	**7.1 & TYN (n=50)**	**0.022**	**1**	**0.065**	**0.0001**

(a)Fisher's exact test; (b) Chi-square test, Hpsb= hyperplasia in suprabasal layers, Hpb=hyperplasia in basal layer

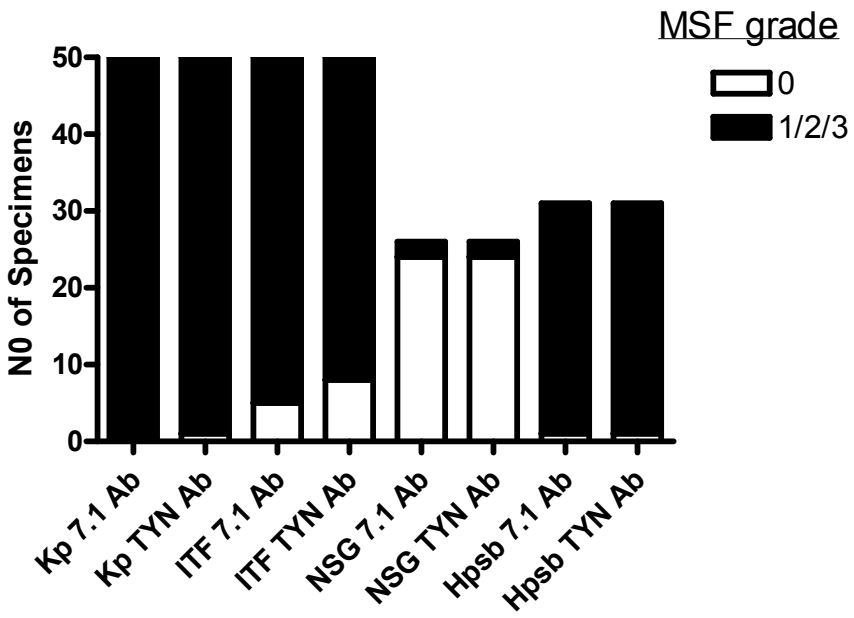


Figure 4.7: Number of OSCC specimens showing the indicated MSF grades of both isoforms. The tissues were stained with antibody 7.1and TYN (n=50) as indicated. The tissues examined included pathological invasive tumour front (ITF) (n=50), keratinpearl (Kp) (n=50), histologically normal salivary gland (NSG, n=26), hyperplasia suprabasal cell layers (Hpsb) (n=31) and hyperplasia basal cell layers (Hpb) (n= 31). Chi square tests (MSF grades 0 v. 1 v. 2/3) and Fisher's Exact test (MSF grades 0 v. 1/2/3) demonstrated statistical tests as shown in (Table 4.2 & 4.3).

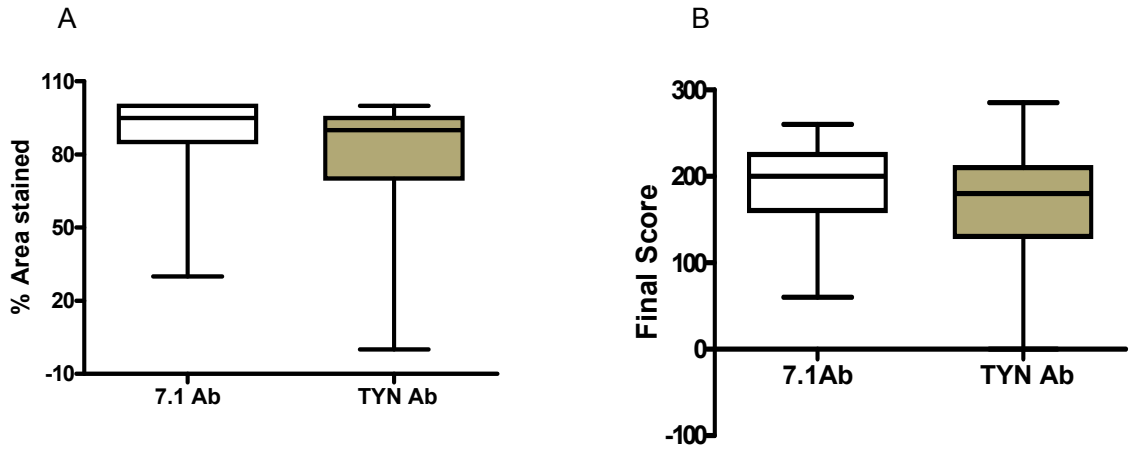


Figure 4.8: Comparison between two MSF isoforms antibodies (7.1 and TYN).
MSF expression in the epithelium of OSCC was evaluated and compared. There were significant differences between the two antibodies in terms of the (A) % of area stained (P <0.008 Mann–Whitney test) and (B) final score (% area x intensity; P <0.016, Mann–Whitney test) respectively.

4.4.5 Survival analysis

Survival data was available for 46 OSCC patients; clinical details of these are presented in Table 4.5. Specimens from the 46 patients were stained and evaluated for MSF expression using 7.1 Ab; TYN Ab was evaluated on 45 specimens. The prognostic value of MSF expression was determined by Kaplan-Meier survival curves and log rank analyses. The results, (summarised in Table 4.6) showed a significant association between high MSF expression in the invasive tumour front (ITF) and poor patient survival. In contrast, MSF expression in the keratinised areas (Kp) was not associated with patient survival. The 5 year overall survival rates for patients with high MSF grades (2/3) in ITF were significantly worse than for those patients with low MSF grades (0/1) (log-rank test: $p< 0.001$ and $p<0.023$ for total MSF and MSF-aa respectively) (Fig 4.9 & 4.10). Among the various MSF indices examined MSF grade (divided 0/1 vs 2/3) was the most informative, although results obtained with MSF

final score and the % area stained also reached statistical significance or near significance in some cases (P= 0.04-0.08) (Table 4.6). Of the two antibodies used, TYN produced more informative results than 7.1 (Table 4.6). Significant differences in overall survival according to MSF grade expression were most apparent at 60 months follow-up time.Tumour-free survival and overall survival were also significantly different at 125 months follow-up time according to MSF grade assessed with TYN antibody. Clinical variables (pathology grade and T, N, Stage grouping) were not associated with patient survival (Table 4.7).

Table 4.5: Clinical details of OSCC patients

Variable	Description	7.1 Ab N =46	TYN Ab N =45
Overall survival (years)	Median	61.5	56.02
	IQR	12-85	12-120
	Mean	55.2	63
	SD	37.47	37.8
Tumour-free survival (years)	Median	46	48.31
	IQR	8.5-60	8.5-120
	Mean	36.9	47
	SD	23.7	37.1
Censor (dead/alive)	(0) dead	16	16
	(1) alive	30	29
Gender	(1) male	39	38
	(2) female	7	7
Age (years)	Median	54	54
	IQR	47-62	48-62
	Mean	54.1	54.2
	SD	10.3	10.4
Pathology grade	1	5	5
	2	36	36
	3	5	4
Pathological. T	1	5	5
	2	29	28
	3	4	4
	4	8	8
Pathological. N	N 0	21	21
	N 1	15	15
	N 2	9	8

Table 4.6: The prognostic significance of the clinical characteristics of OSCC stained by ant-MSF (Ab 7.1) as derived from a long rank test

Experimental index.	Clinical index	Division	Ab giving significant differences	(p value) 7.1	(p value) TYN
				n=46	n=45
MSF grade	O.S- ITF 125 months	0/1 v 2	TYN>7.1?	P=0.057	P=0.010*
MSF grade	O.S- ITF 48 months	0/1 v 2	7.1>TYN?	P= 0.004*	P= 0.065
MSF grade	O.S- ITF 60 months	0/1 v 2	TYN & 7.1*	P= 0.001*	P= 0.023*
MSF grade	TFSurv-ITF 125 months	0/1 v 2	TYN>7.1	P=0.113	P=0.004
MSF grade	TFSurv-ITF 48 months	0/1 v 2	TYN>7.1?	P= 0.062	P= 0.049
MSF grade	TFSurv-ITF 60 months	0/1 v 2	TYN>7.1?	P= 0.089	P= 0.027*
MSF grade	O.S- Kp 125 months	0/1 v 2		P=0.276	P=0.275
MSF grade	O.S- Kp 60 months	0/1 v 2		P=0.428	P=0.437
MSF grade	TFsurv- Kp 125 months	0/1 v 2		P=0.286	P=0.291
MSF grade	TFsurv- Kp 60 months	0/1 v 2		P=0.340	P=0.346
% area stained	O.S 125	medians		P=0.576	P=0.491
% area stained	O.S 48 months	medians		P= 0.909	P= 0.205
% area stained	O.S 60 months	medians		p=0.825	p=0.194
% area stained	TFsurv 125 months	medians		p=0.964	p=0.302
% area stained	TFsurv – 48 months	medians		p=0.685	p=0.113
% area stained	TFsurv – 60 month	medians	TYN>7.1	p=0.634	p=0.060
Final score	O.S	medians		p=0.560	p=0.330

	125				
Final score	**O.S 48 months**	**medians**	**7.1>TYN?**	**p=0.041***	**p=0.076**
Final score	**O.S 60 months**	**medians**	**TYN?>7.1?**	**p=0.085**	**p=0.063**
Final score	**TFsurv 125 months**	**medians**		**p=0.682**	**p=0.340**
Final score	**TFsurv – 48 months**	**medians**		**p=0.449**	**p=0.412**
Final score	**TFsurv – 60 month**	**medians**		**p=0.515**	**p=0.489**

O.S= overall survival, TFsur= tumour free survival, ? = significant at the 90% level of Confidence, * = significant differences

Table 4.7: The relationship between clinical characteristics (Path-grade, Path-T and Path-N) and overall and tumour free survival rates as derived from Kaplan-Meier Test.

Experimental index.	**Clinical index**	**Division**	**(p value)**	**Test/comments**
Path- grade	**OS – 125 months**	**1 v 2 v 3**	**p=0.643**	**Log rank test**
Path- grade	**OS – 125 months**	**1 v 2/3**	**p=0.346**	**Log rank test**
Path-grade	**TFsurv 125 months**	**1 v 2 v 3**	**p=0.601**	**Log rank test**
Path-grade	**TFsurv 125 months**	**1/2 v 3**	**p=0.860**	**Log rank test**
Path-T	**OS – 125 months**	**1 v 2 v 3 v 4**	**p=0.360**	**Log rank test**
Path-T	**TFsurv 125 months**	**1 v 2 v 3 v 4**	**p=0.388**	**Log rank test**
Path-N	**OS – 125 months**	**0 v1 v 2**	**p=0.206**	**Log rank test**
Path-N	**TFsurv 125 months**	**0 v1 v 2**	**p=0.337**	**Log rank test**

Path = pathological, T = size, N = lymphnode involvment

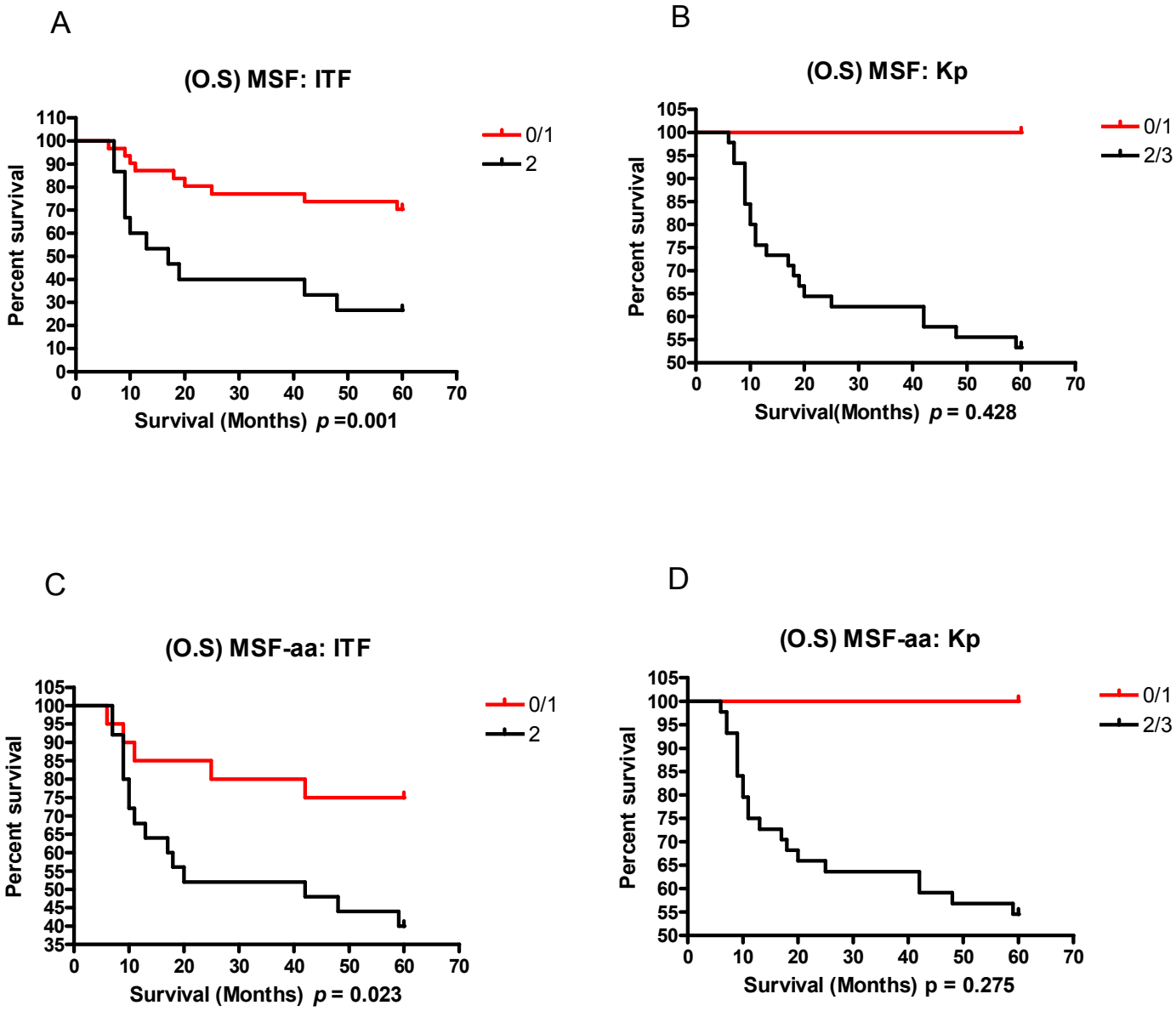


Figure 4.9: Correlation between MSF isoforms expression and survival rate in patients with OSCC.

as determined by the Kaplan–Meier method. The overall survival of patients with high expression of MSF was statistically worse than that of those with no/ low MSF expression (log-rank; $p<0.001$, 0.023). No association was found in the keratin pearl (Kp) for both isoforms.

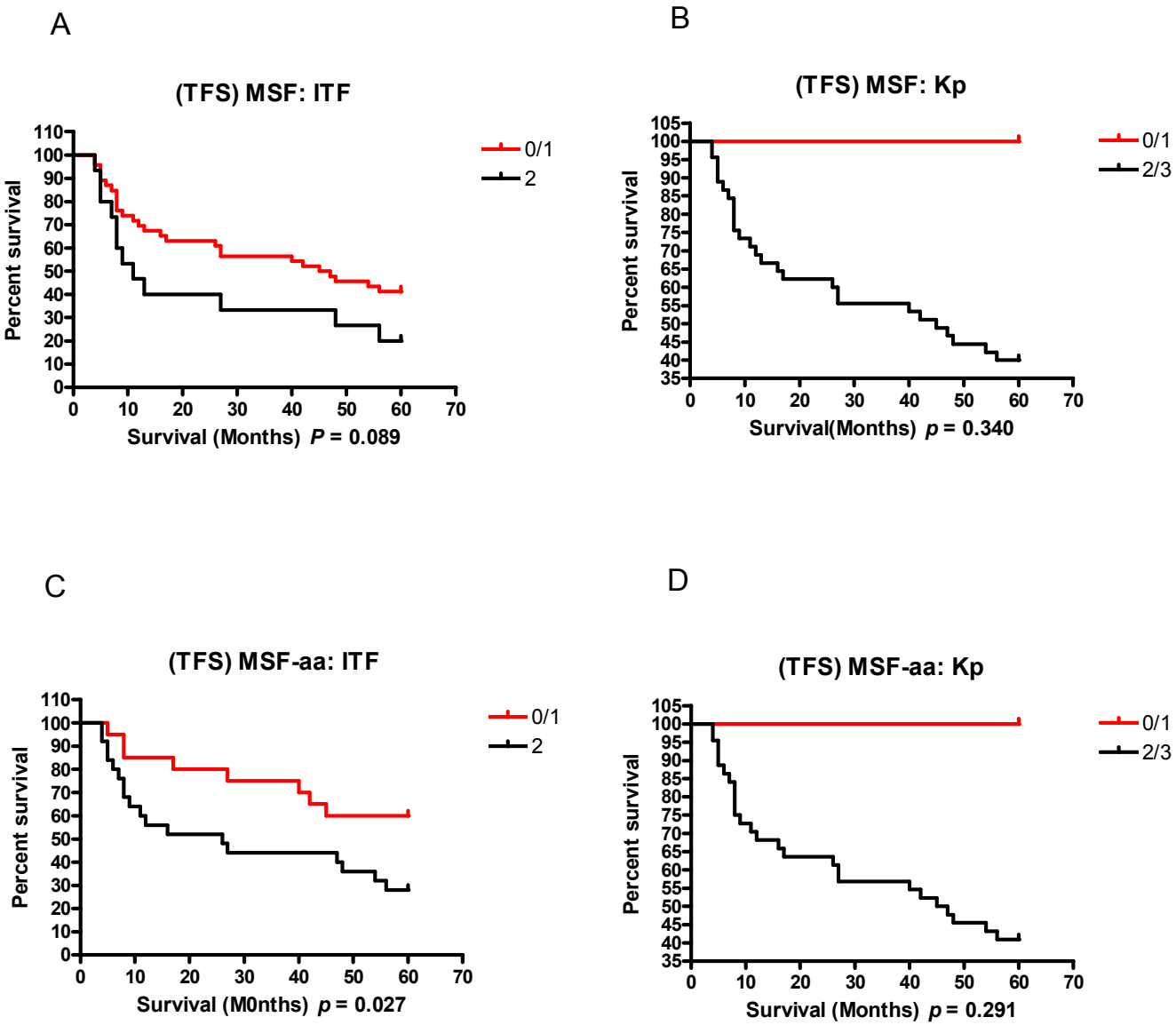


Figure 4.10: Correlation between MSF isoforms expression and tumour free survival rate in the OSCC patients.

as determined by the Kaplan–Meier method. The disease (tumour)-free survival of patients with expression of MSF-aa was statistically worse than that of those with low MSF expression (log-rank; $p < 0.027$). Patients with highly expression of total MSF was trend but not significant. No association was found in the keratin pearl (Kp) for both isoforms.

4.5 Discussion

Staining of formalin-fixed paraffin-embedded specimens by immunohistochemistry (IHC) is commonly used, as the specimens can be stored for a long time, thereby allowing retrospective studies of a large population. The identification of novel biomarkers in oral squamous cell carcinoma (OSCC) is aimed at improving the diagnostic and or prognostic indicators in these tumours and evaluating the possible functional importance of molecules that may become the target for new therapies. In the past few years, significant progress has been made in the identification and understanding of prognostic biomarkers involved in predicting OSCC aggressiveness (Chapter one introduction). The TNM classification system cannot predict the biological features of tumour cells and, therefore, is unable to individualise the prognosis. The identification of novel biomarkers could help in definitive treatment planning and improve survival consequently.

Important molecular interactions that enhance or inhibit tumour progression occur at the tumour-host interface (Bryne *et al.,* 1998). It had been suggested that the invasive tumour front (ITF) comprises the most aggressive cells, which have the capacity to invade neighbouring tissue structures, including vessels, and thereby metastasise. Previous studies have reported that the characteristics of the ITF are most important for the diagnosis and prognosis of oral cancer (Bryne *et al.,* 1998; Bankfalve and Piffko, 2000). Interactions between stromal and tumour cells at the ITF are expected to play a significant role in modulating tumour progression. Fibroblasts and endothelial cells are the main stromal cells involved in these interactions; changes in the behaviour of these stromal cells at the ITF, are reflected by displaying a number of fetal characteristics, this may therefore provide independent prognostic indices (Bankfavi and Piffko, 2002).

In the present study, the expression of MSF in OSCC tissue sections was quantified using IHC and two different antibodies: 7.1, and TYN. As shown in chapter three, Ab7.1 recognises MSF+aa and MSF-aa whereas TYN recognises MSF-aa only. The ITF and the centre of the tumour, including keratin pearls (Kp) were assessed independently. The results may be summarised as follows:

- Both antibodies stained most OSCC specimens (100 % and 98% positive specimens with 7.1 and TYN, respectively). Staining was

observed, to different extents, in carcinoma cells and associated stromal cells.

- In adjacent non-tumour tissue, staining was observed, to different extents, in NSG and Hp. Staining was restricted to epithelial ductal cells in the NSG and to the Hpsb area of Hp.
- Some specimens showed positive staining with 7.1 and negative with TYN, the reverse pattern was not observed.
- The percentage of MSF-positive specimens followed a pattern, represented as NSG=Hpb<Hpsb<ITF<Kp, for TYN Ab and as NSG=Hpb< Hpsb=ITF<Kp for 7.1 Ab.
- High MSF expression in the invasive tumour front (ITF) was significantly associated with shorter patient survival. In contrast, MSF expression in the keratinised areas (Kp) was not associated with patient survival. More informative results were obtained with TYN antibody than with 7.1 antibody.

Differences in staining between ITF and Kp areas has been noted previously regarding the expression of different molecules, Wang et al, (2009) reported that E-cadherin expression at the ITF is lower than that in centre/superficial part for most of OSCC. E-cadherin at the ITF is statistically associated with poor survival of OSCC patients.

MSF exhibits an oncofoetal pattern of expression as defined by its (*i*) constitutive production by keratinocytes, fibroblasts and endothelial cells in foetal skin, (*ii*) reduced or undetectable expression in skin and other tissues in healthy adults, and (*iii*) re-expression by both carcinoma and stromal cells in the majority of common human tumours, including those of the breast, lung, colon, prostate and oral mucosa (Schor *et al.,* 2003 and unpublished observations). This study demonstrates that MSF is expressed by carcinoma and stromal cells in OSCC. Re-expression of MSF in previously negative epithelium during malignant transformation might be regarded as a return to an embryonic expression pattern. The foetal-like characteristics of the cells at the ITF have been previously noted (Bankfavi and Piffko, 2002).

Results presented here suggest that a small number of OSCC specimens express MSF+aa only. However, most OSCC specimens express either MSF-aa only or a mixture of MSF+aa and MSF-aa. In chapter three it was demonstrated that

NGAL inhibits MSF+aa, but not MSF-aa. Since NGAL is also expressed in OSCC and Hp (results not shown), it is speculated that MSF-aa is the most relevant MSF isoform in terms of diagnosis, prognosis and functionality.

In agreement with this hypothesis, the results presented in this chapter indicate that MSF expression assessed with TYN antibody is more informative than results obtained with 7.1 antibody regarding the prognosis of OSCC patients. With both antibodies, high MSF expression at the ITF was significantly associated with shorter patient survival.

4.6 Conclusion

- Data presented here provide an initial indication that MSF isoforms expression is up-regulated in both the epithelial and stromal cell compartments of OSCC.

- This is the first report that the expression of MSF is a novel independent poor prognostic factor in OSCC. Therefore, MSF is a useful marker for choosing therapy and for predicting the poor outcome of patients with OSCC. Patients with OSCC who show positive MSF expression should be followed-up carefully. In addition, MSF might be a strong candidate to target for future therapy in OSCC.

4.7 Further work

- To assess the presence of MSF message in OSCC by using in situ hybridization

- To study the expression of MSF in the normal oral mucosa and epithelial dysplastic tissue and compare it with the result of MSF in OSCC, to provide a rational platform for subsequent more extensive investigation of the possible diagnostic significance of MSF expression in OSCC.

- To examine a larger number of patients

5. Chapter five: MSF Expression in Salivary gland tumours

5.1 Introduction:

Salivary gland tumours (SGT) are relatively rare lesions, accounting for only 3–6% of all head and neck neoplasms. They are a morphologically and clinically diverse group which present a challenge to head and neck surgeons and pathologists, both in terms of diagnosis and clinical management. The majority of tumours arising from the minor salivary glands are malignant (Speight and Barrett, 2002; WHO Classification of Tumours, 2005; Ward and Levine, 1998). The identification and validation of novel stratification and predictive biomarkers for SGT would consequently be of significant clinical utility. Migration-stimulating factor (MSF), a soluble genetically truncated isoform of fibronectin, is a potent oncofoetal regulatory molecule. The expression of MSF in salivary gland tumours has not been examined previously; it will be of great clinical value to identify effective early markers such as MSF for the diagnosis and prognosis of salivary gland tumours.

5.2 Aims:

The aims of this study have been to ascertain the possible presence, diagnostic significance and role of MSF in salivary gland tumours. Immunohistochemistry was used to compare the expression pattern of MSF in histologically normal salivary gland tissue adjacent to benign and malignant SGT.

5.3 Materials and Methods:

5.3.1 Specimens: SGT study

Formalin-fixed, paraffin-embedded, archival tissue specimens of benign (n=7) malignant (n=27) minor salivary gland tumours (SGT) were obtained from the Domagk-Institute of Pathology, University Clinic of Munster, Germany. The majority of the malignant tumours were adenoid-cystic carcinomas (n=16) and mucoepidermoid carcinomas (n=6). The majority of the benign tumours (6) were pleomorphic adenomas. The anatomical location of the tumours and relevant patient information are presented in Table 5.1. Sixteen of the tumour specimens (15 malignant and 1 benign) included regions of adjacent histologically normal salivary gland (NSG), as indicated in Table 5.2.

5.3.2 Immunohistochemistry:

Tissues were stained with two monoclonal antibodies: identification antibody 7.1, that recognise the MSF-unique sequence and antibody TYN that recognises

MSF–aa but not MSF+aa (as described in Chapter three characterisation of identification antibodies to MSF). MSF expression in paraffin-embedded archival specimens of salivary gland tumours was examined by standard immunohistochemistry as outlined in chapter two (Materials and Methods).

Table 5.1: Clinical characteristics of the malignant and benign salivary gland tumours examined were stained by two antibodies (7.1 and TYN). Malignant tumours included: Adenoid cystic carcinomas (ACC stained by 7,1 Ab n=16 and by TYN Ab n=13 , three missing 2 in upper jaw and 1 in palate) , mucoepidermoid carcinomas (MC), adenocarcinomas (AC), basal cell adenocarcinoma (BcAC), sebaceous adenocarcinoma (SA, SC), Benign tumours included pleomorphic adenoma (PA) and angiolieomyoma (Ang).

Tumour	n	Anatomical location								Age Average (range)	Gender (F:M)
		lip	Oral mucosa	Upper Jaw	palate	cheek	Lower jaw	Sub Mand	Floor of the mouth		
ACC	**16**	**1**	**1**	**8**	**3**	**1**	**1**		**1**	**57.1 (35-83)**	**13:3**
MC	**6**			**2**	**2**	**1**		**1**		**47.5 (32-66)**	**2:4**
AC	**2**		**1**	**1**						**43.5 (36-51)**	**1:1**
BcAC	**2**				**1**	**1**				**58-59**	**0:2**
SA	**1**			**1**						**79**	**1:0**
All malignant	**27**	**1**	**2**	**12**	**6**	**3**	**1**	**1**	**1**	**55.7 (32-83)**	**17:10**
PA	**6**			**2**	**1**	**1**		**2**		**41.6 (20 -75)**	**4:2**
Ang	**1**				**1**					**29**	**0:1**
All benign	**7**			**2**	**2**	**1**		**2**		**39.8 (20 -75)**	**4:3**
All tumours	**34**	**1**	**2**	**14**	**8**	**4**	**1**	**3**	**1**	**52.5 (20-83)**	**21:13**

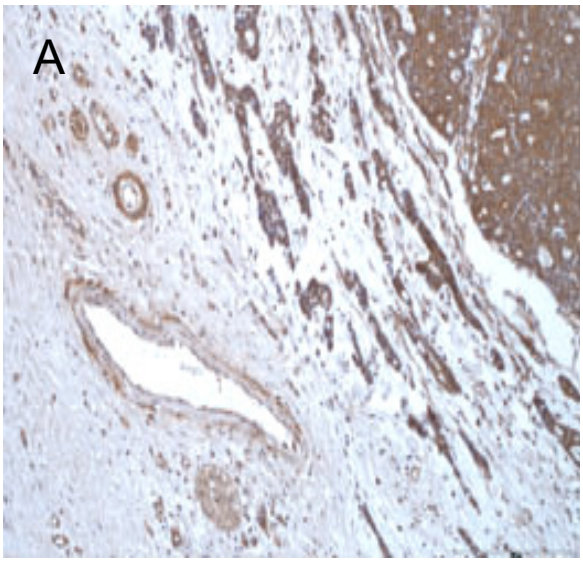
Table 5.2: Tumour specimens containing histologically normal salivary glands adjacent to the tumour. Normal salivary gland (NSG) was found adjacent to adenoid cystic carcinoma (n=9 ACC stained by 7.1 Ab; and n=7 by TYN Ab), mucoepidermoid carcinoma (MC), adenocarcinoma not otherwise specified (AC), basal cell adenocarcinoma (BcAC) and pleomorphic adenoma (PA).

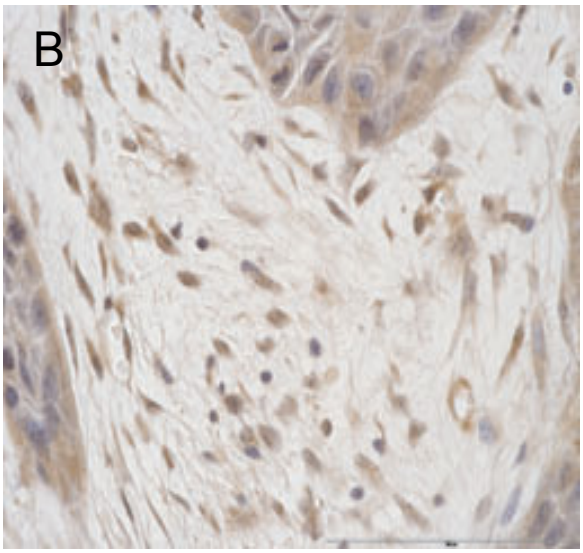
Adjacent tumour	n	Anatomical location							Age Average (range)	Gender (F:M)
		lip	Oral mucosa	Upper Jaw	palate	cheek	Lower jaw	Sub Mand		
ACC	**9**	**1**		**6**	**1**	**1**			**58 (35-80)**	**8:1**
MC	**3**			**1**	**1**			**1**	**41.3 (32-59)**	**1:2**
AC	**2**		**1**	**1**					**43.5 (36-51)**	**1:1**
BcAC	**1**					**1**			**58**	**0:1**
All malignant with adjacent NSG	**15**	**1**	**1**	**8**	**2**	**2**		**1**	**50.2 (32-80)**	**10:5**
Benign (PA) with adjacent NSG	**1**			**1**					**36**	**1:0**
All tumours	**16**	**1**	**1**	**9**	**2**	**2**		**1**	**43.1 (32-80)**	**11:5**

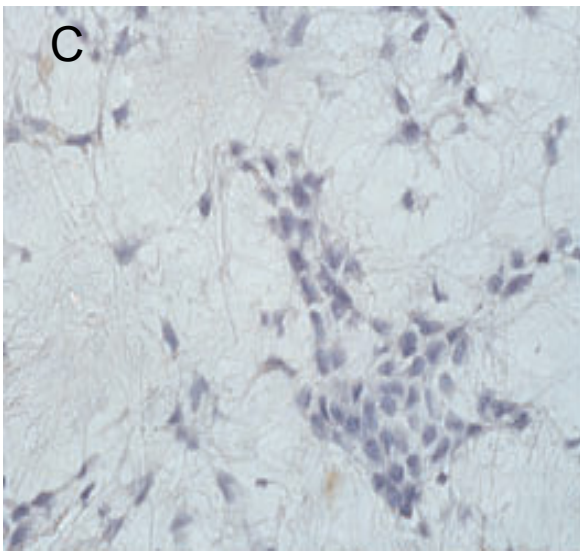
5.4 Results:

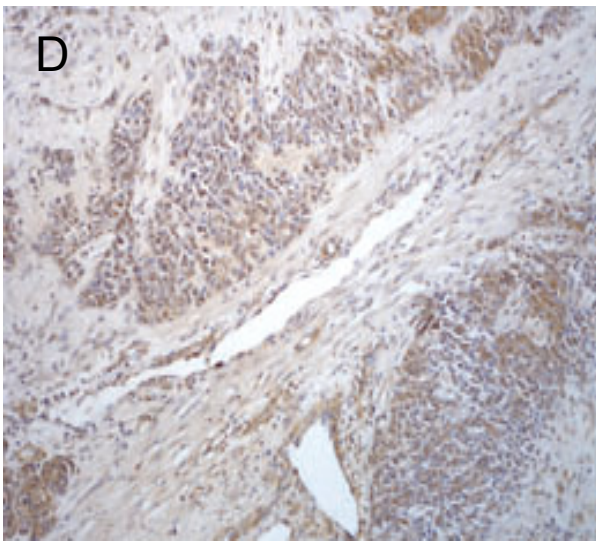
5.4.1 Immunolocalisation of total MSF

Duplicate sections of benign and malignant small salivary gland tumours (SGT) (Table5.1) were stained with MSF-specific antibody mab7.1 (Schor *et al.,* 2003). Histologically NSG was presented adjacent to some of the tumours (Table 5.2). MSF was differentially expressed in the salivary gland tissues examined. Positive staining was observed in the majority of malignant tumours, both in the tumour and in the associated stromal cells. Representative examples of MSF expression by malignant tumour cells and tumour-associated stromal cells are presented in (Fig 5.1A, B). Examples of negative and positive benign tumours were also encountered (Fig 5.1C, D). Positively stained acinar cells were rarely present in NSG (Fig 5.1E, F). Positively stained inflammatory cells were occasionally detected in association with malignant tumours (Fig 5.1G). Sections incubated with normal mouse IgG, instead of MSF antibody, showed no staining (Fig 5.1H).









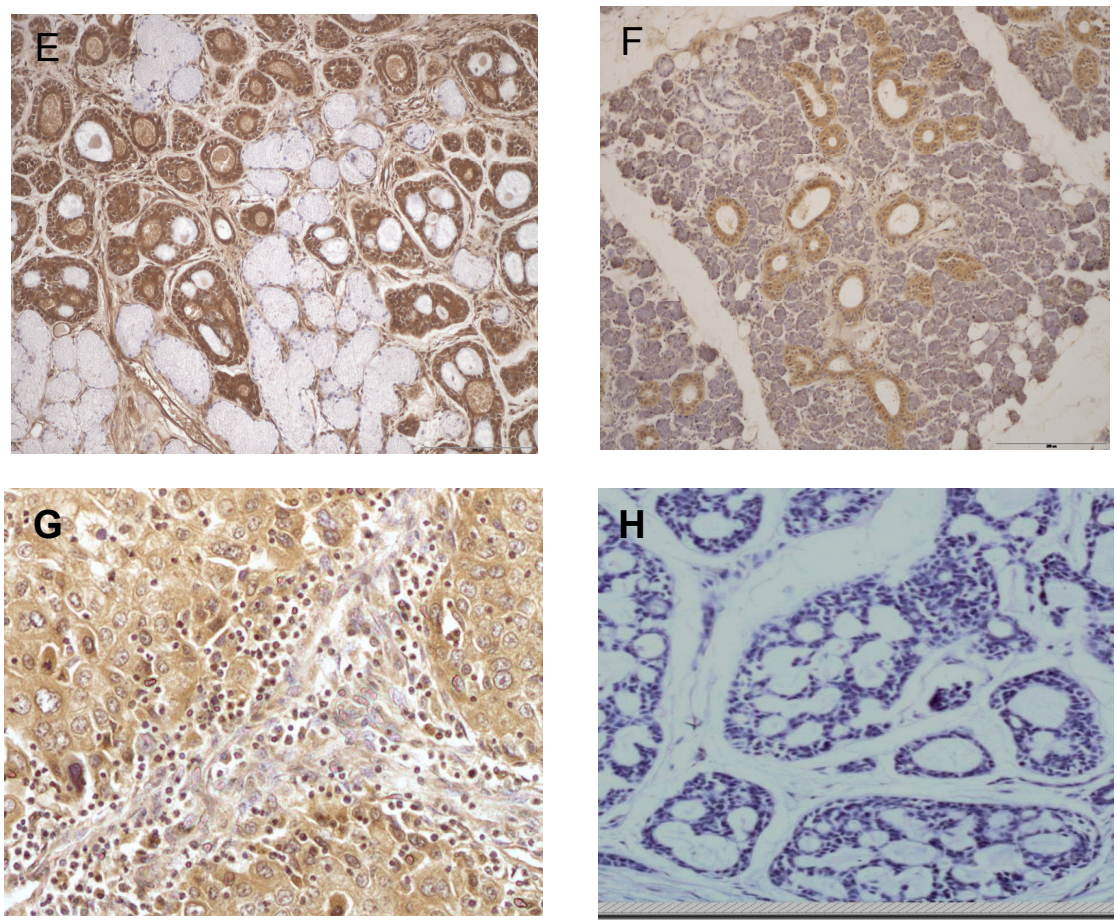


Figure 5.1: Immunolocalisation of total MSF in salivary gland tumours.
(A, B) MSF expression in malignant tumours, adenoid cystic carcinoma (A) and mucoepidermoid carcinoma (B) showing MSF staining in the tumour cells and associated blood vessels and fibroblasts. (C) Negative MSF staining in benign tumour (pleomorphic adenoma). (D) MSF-positive pleomorphic adenoma. (E) Adenoid cystic carcinoma showing negative MSF staining in histologically normal salivary gland (NSG) (mucous cells) adjacent to positive tumour. (F) Positive excretory and striated ducts next to negative serous cells in NSG. (G) Positive inflammatory cells and carcinoma cells in a mucoepidermoid carcinoma. (H) Sections incubated with normal mouse IgG, instead of MSF antibody, showed no staining (negative controls Original photographs were taken at magnification x 100 (A, D, E, F, G, H) or x 400 (B, C).

5.4.2 Differential expression of total MSF in salivary gland tissues

MSF expression was evaluated in NSG (n = 16), benign SGT (B; n = 7) and malignant SGT (M; n = 27). The group of benign SGT consisted of six epithelial-derived tumours (pleomorphic adenomas) and one stromal-derived tumour (angiomyoma). MSF expression was first graded as negative (0), weak positive (1), moderate (2) or strong positive (3) by four independent observers. To compare the different tissues, results are presented in Fig 5.2A as the percentage of specimens showing the various MSF grades. This initial classification (overall MSF grade) indicated that a significantly greater proportion of the malignant tumours were positively stained for MSF compared with benign lesions or NSG. A significant difference was also observed between benign tumours and NSG. Therefore, overall MSF expression increased significantly in a step-wise fashion from normal salivary gland to benign and malignant tumours ($P < 0.006$-0.0001, Fig 5.2A); with moderate / strong positive specimens representing 6%, 29% and 81% of the normal, benign and malignant specimens, respectively. The inclusion of (angiomyoma) brought the percentage of moderate / strong positive specimens to 33%, but did not alter the significance of the results ($P<0.005$–0.001). Significant differences between pleomorphic adenomas and malignant tumours were also observed even when comparing specific sub-groups of the latter (Table 5.1), such as adenoid cystic carcinomas ($P<0.045$) and mucoepidermoid carcinomas ($P<0.049$), in spite of the small number of specimens involved. Within the group of SGT specimens examined, MSF expression was not related to the anatomical site of origin or to the age or gender of the patient. The overall MSF grade (Fig 5.3A) gives a general assessment of the sections, including epithelial and stromal compartments. More detailed observations of the tumours revealed that MSF was heterogeneously distributed within both the tumour and stromal compartments (such as fibroblasts, blood vessels and inflammatory cells), with a greater proportion of the specimens being positively stained in malignant than in benign tumours in all cellular compartments (Fig 5.3A). A benign tumour derived from the vascular smooth muscle cells (angiomyoma) was positive for MSF, whereas the blood vessels of the normal salivary gland were MSF-negative. Within the epithelium, (Fig 5.5) significant differences between benign and malignant tumours occurred in terms of highest (hot spot) intensity, percentage of area stained and final score, (i.e. area x intensity). Overall MSF grade of the NSG reflects mainly the staining present in the acinar cells. More detailed observation of the

different NSG cell types indicated that mucous and serous acinar cells were indeed predominantly negative for MSF, whereas ductal cells commonly exhibited a diffuse positivity, this being stronger in the luminal cells compared with basal and myoepithelial cells (Fig 5.1E, F). Of 16 NSG specimens examined, 10, 11 and 15 (63%, 69% and 94%) showed positive intercalated, striated and excretory salivary ducts, respectively. Only one specimen (6%) showed MSF staining in acinar (serous) cells and nine (56%) in myoepithelial cells (Table 5.3). A comparison of NSG adjacent to SGT (as above) and NSG adjacent to OSCC (chapter 4) revealed no significant differences regarding MSF expression (Table 5.3).

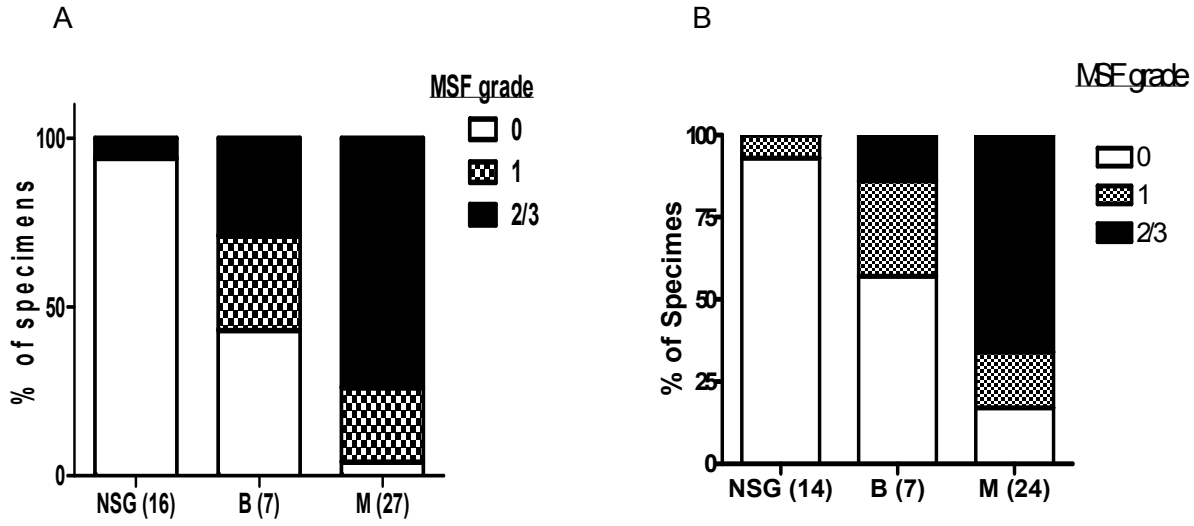


Figure 5.2: Percentage of SGT specimens showing the indicated MSF isofroms grades.
The tissues were stained with antibody 7.1(A) and TYN (B), as indicated. The tissues examined included histologically normal salivary gland (NSG, n=16 or 14), benign (B, n=7) and malignant salivary gland tumours (M, n=27 or 24). Chi square tests (MSF grades 0 v. 1 v. 2/3) and Fisher's Exact test (MSF grades 0 v. 1/2/3) demonstrated significant differences between M and B ($p<0.006$-0.021 0.01-0.006 for 7.1 and for TYN 0.037-0.0520.02-0.03), between M and NSG ($p< 0.0001$) for both antibodies and between B and NSG ($p<0.017$ 0.04) for antibody 7.1, whilst no significant differences between B and NSG for TYN antibody. All p values were $p< 0.0001$ when the percentages of specimens (as shown in the graph) were compared.

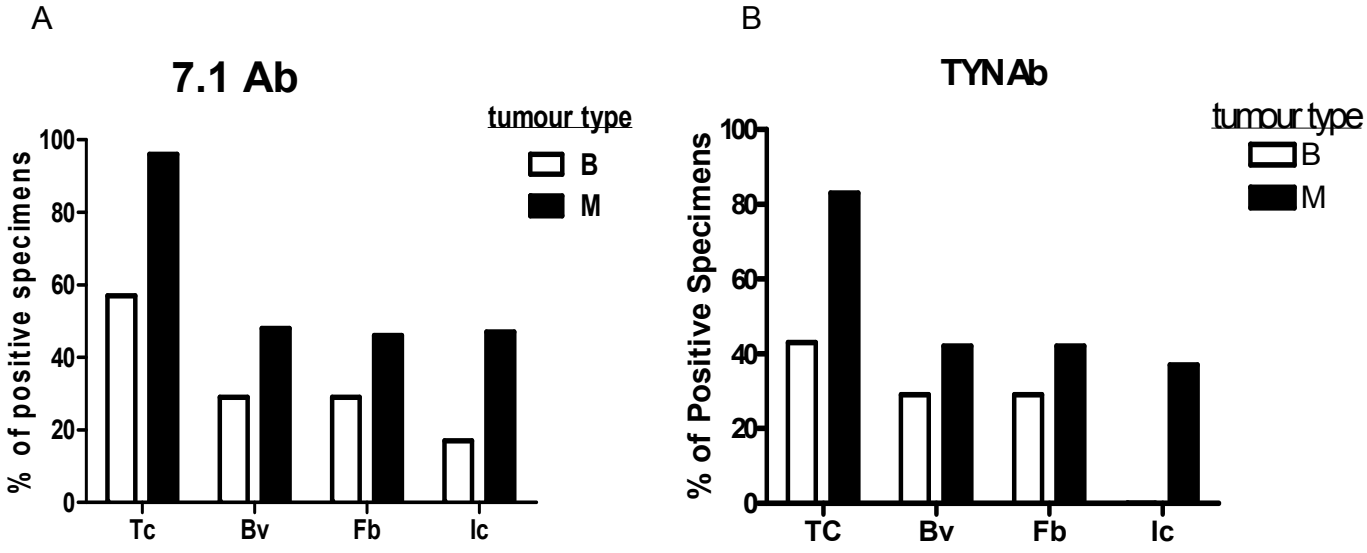
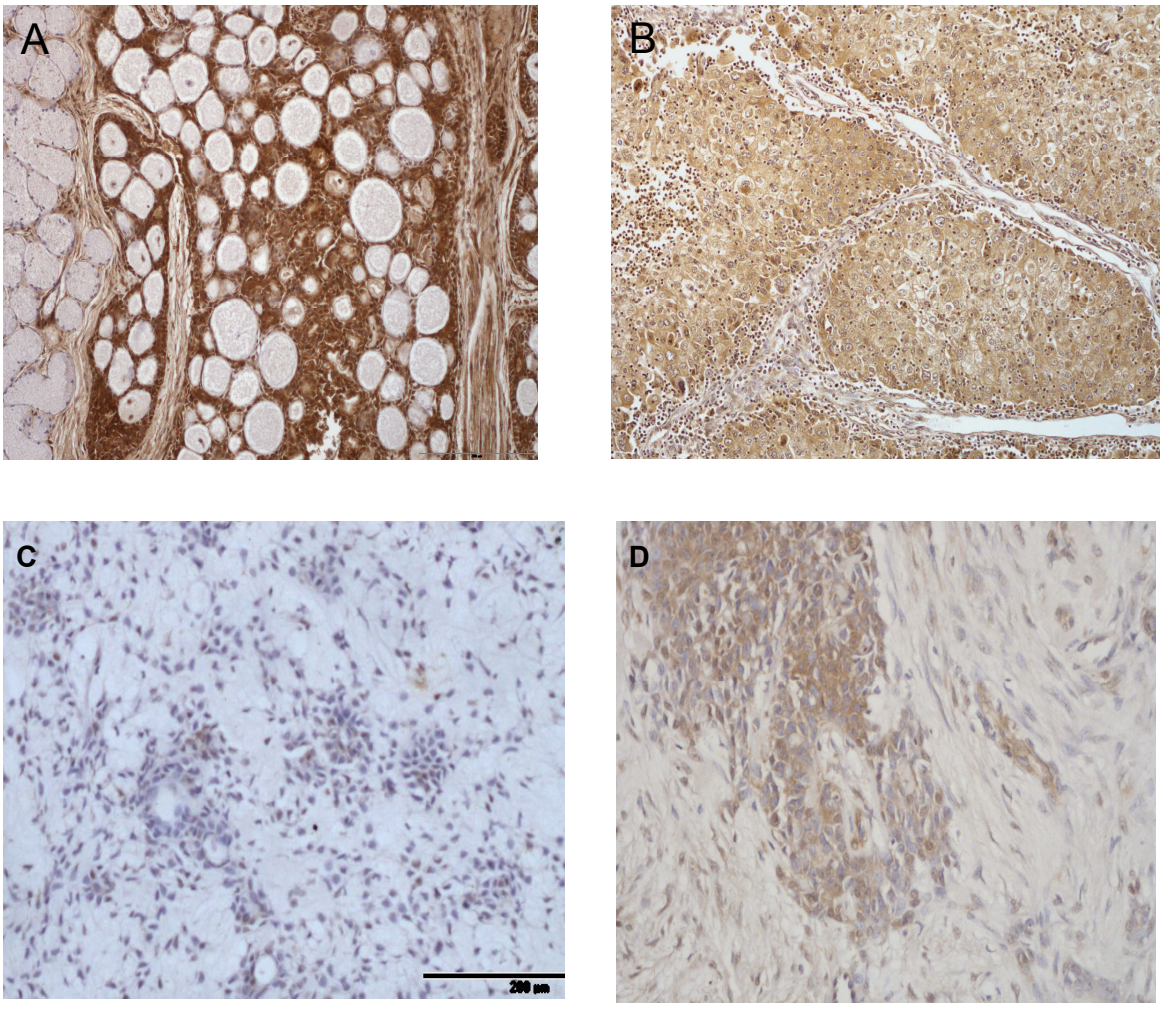


Figure 5.3: Epithelial and stromal MSF expression in benign (B) and Malignant (M) salivary gland tumours.

Results show the percentage of specimens graded positive (grades 1/2/3) stained by antibody 7.1 (A) and TYN (B) for different cellular compartments, including tumour epithelial cells (TC), blood vessels (Bv), fibroblasts (Fb); M=24 and B=7 and inflammatory cells (Ic; M=19 and B=7). The difference between malignant and benign tumours was analysed by Fisher's exact test for the actual number of specimens assessed (n) and for the percentage of specimens (%, as shown in the graph). The respective p values for (n) and (%) were: 0.02 and 0.001 for Tc (7.1 Ab) and 0.05 - 0.001 for (TYN Ab); 0.19 and 0.0001 for Bv (7.1 Ab) and 0.675 - 0.075 for (TYN Ab); 0.19 and 0.0001 for Fb (7.1 Ab) and 0.675-0.075 for (TYN Ab); 0.178 and 0.0001 for Ic (7.1 Ab) and 0.133-0.0001 for (TYN Ab).

5.4.3 Immunolocalisation of MSF-aa

Following the same standard immunohistochemical procedures and the same methods of assessment for mab7.1 specific total MSF antibody, 31 duplicate sections of benign and malignant small salivary gland tumours (SGT) were stained with mouse monoclonal antibody specific to MSF-aa (TYN1.2) (Chapter 4). Histologically NSG was present adjacent to some of the tumours. MSF was differentially expressed in the salivary gland tissues examined. Positive staining was observed in the majority of malignant tumours, both in the tumour and in the associated stromal cells (Fig 5.4).



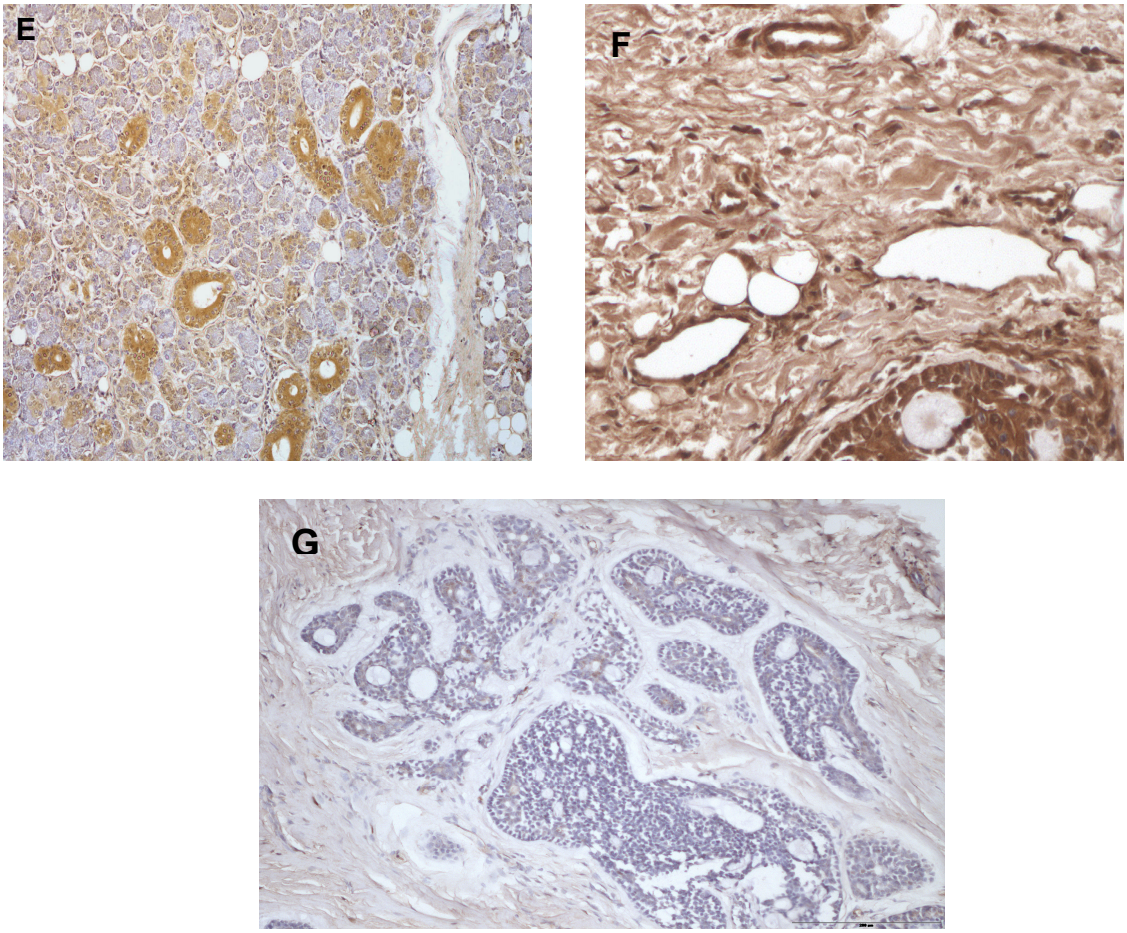


Figure 5.4: Immunolocalisation of MSF-aa in the salivary gland tumours.
(A, B) MSF expression in malignant tumours, adenoid cystic carcinoma adjacent to negative NSG (A) and mucoepidermoid carcinoma (B) Mucoepidermoid carcinoma (C) Negative MSF staining in benign tumour (pleomorphic adenoma). (D) MSF-positive pleomorphic adenoma. (E) Positive excretory and striated ducts next to negative serous cells in NSG. (F) Showing MSF staining in the tumour cells and associated blood vessels and fibroblasts.(G) Sections incubated with normal mouse IgG, instead of MSF antibody, showed no staining (negative controls). Original photographs were taken at magnification x100 (A, B, C, D, E, F and G)

5.4.4 Differential expression of MSF-aa in salivary gland tissues

Differential expression of MSF in salivary gland tissues was evaluated in NSG (n = 14), benign SGT (B; n = 7) and malignant SGT (M; n = 24). MSF-aa expression was graded in a similar way with mab 7.1, by two independent observers. To compare the different tissues, results are presented in (Fig 5.2B) as the percentage of specimens showing the various MSF-aa grades. This initial classification (overall MSF-aa grade) indicated that a significantly greater proportion of the malignant tumours were

positively stained for MSF-aa compared with benign lesions (P< 0.037) or NSG (P< 0.0001). There was no significant difference observed between benign tumours and NSG (Fig 5.2B). The results presented in (Fig 5.5) indicate significant differences between benign and malignant tumours within the epithelium in terms of highest (hot spot) intensity, percentage of area stained and final score, (i.e. area x intensity) (P< 0.028, P< 0.042 and P< 0.027) respectively. The overall MSF-aa grade gives a general assessment of the sections, including epithelial and stromal compartments. More detailed observations of the tumours revealed that MSF-aa was heterogeneously distributed within both the tumour and stromal compartments (fibroblasts, blood vessels and inflammatory cells), with a greater proportion of the specimens being positively stained in malignant than in benign tumours in all cellular compartments (Fig 5.3B)

Overall MSF grade of the NSG reflects mainly the staining present in the acinar cells. More detailed observation of the different NSG cell types indicated that mucous and serous acinar cells were indeed predominantly negative for MSF, whereas ductal cells commonly exhibited a diffuse positivity, this being stronger in the luminal cells compared with basal and myoepithelial cells. Of 14 NSG specimens examined, 2, 9 and 12 (14%, 64% and 86%) showed positive intercalated, striated and excretory salivary ducts, respectively. Only one specimen (7%) showed MSF staining in acinar (serous) cells and seven (50%) in myoepithelial cells (Table 5.3).

Table 5.3: Number and percentage of normal salivary gland specimens adjacent to both SGT and OSCC which are graded as MSF positive for Ab 7.1 and Ab TYN

	Ad OSCC	Ad SGT	Ad OSCC	Ad SGT
Cell type	**Positive 7.1 (n=31)**	**Positive 7.1 (n=16)**	**Positive TYN (n=26)**	**Positive TYN (n=14)**
serous and mucous	**3 (5%)**	**1(6%)**	**2 (8%)**	**0/14**
myoepithelial	**16 (52%)**	**9(56%)**	**10 (38%)**	**7(50%)**
ICD	**18 (58%)**	**10(63%)**	**15 (58%)**	**2(14%)***
Sd	**23 (74%)**	**11 (69%)**	**20 (77%)**	**9 (64%)**
Exd	**28 (90%)**	**15(94%)**	**24 (92%)**	**12(86%)**

Ad= adjacent, ICD=intercalated ducts, Sd =striated duct, Exd=excretory duct

* Positive in the MC 1/6 & AC 1/2 and negative in the rest.

5.4.4 Comparison between 7.1 and TYN Antibodies:

The immunohistochemical identification of total MSF and MSF-aa in sections of paraffin embedded salivary gland tumour was performed using two antibodies (Mouse Monoclonal: mab 7.1 for total MSF and mab TYN 1.1 for MSF-aa). Most of the specimens stained positively for both antibodies. Both antibodies produced intense staining of epithelial tumours, and there was no statistically significant differences between the two antibodies for the total samples including epithelial tumours (benign v benign and malignant v malignant) regarding percentage of area stained, final score and highest intensity of staining (Fig 5.6). Similarly, data present in (Fig 5.3C) shows no significant differences were found between the two antibodies regarding stromal cells compartment.

No significant difference was detected between these antibodies among normal salivary ductal epithelial cell types except for ACC the results indicated that MSF-aa was not expressed in the intercalated duct.

The similar staining pattern of both antibodies suggests that the MSF-aa is the predominant form of MSF in the salivary gland tumours. However, we can-not exclude expression of MSF +aa.

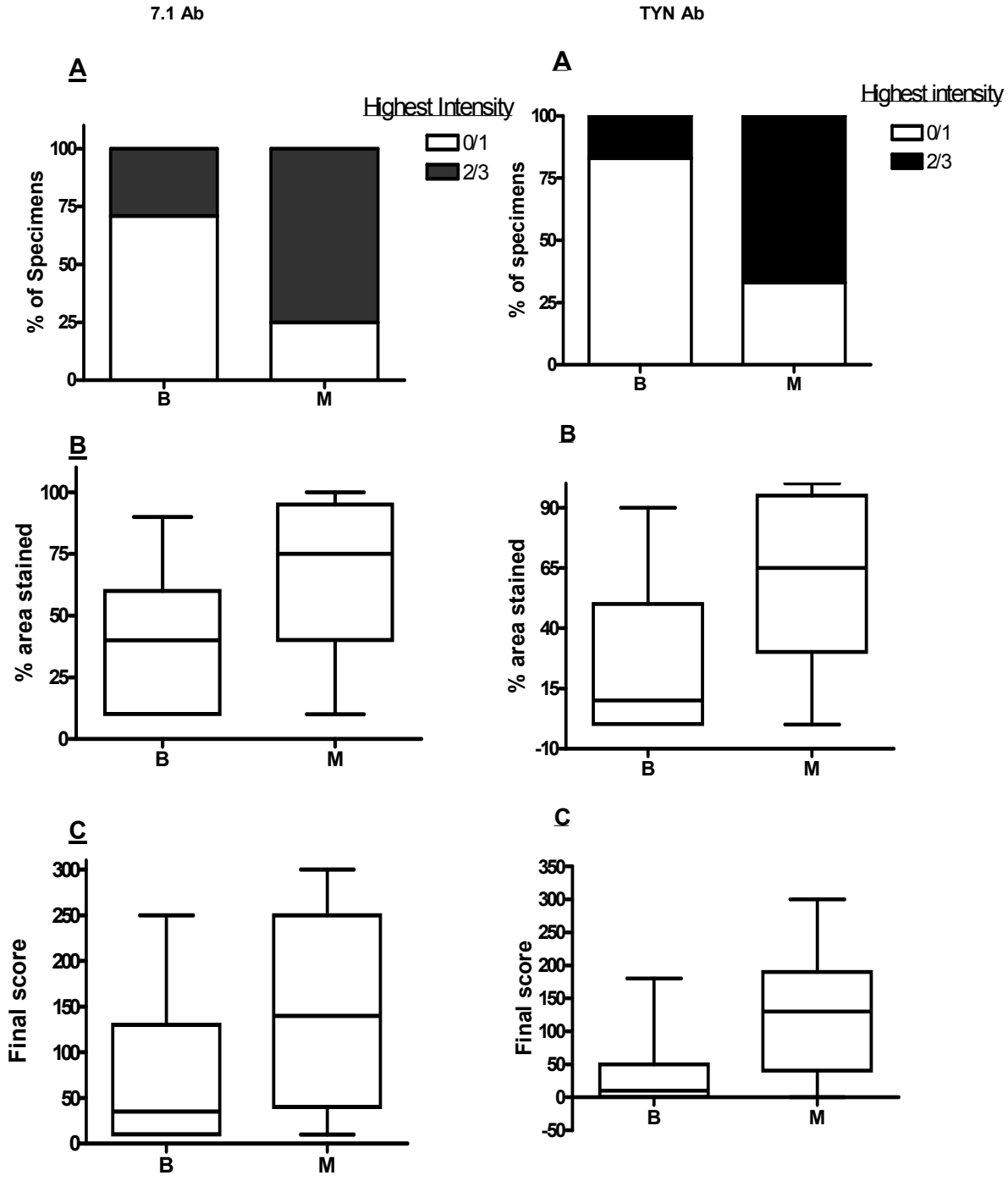


Figure 5.5: Epithelial MSF expression in salivary gland tumours.
The tissues were stained with antibody 7.1 and TYN, as indicated. MSF expression in the epithelium of benign (B; n = 7) and malignant (M; n = 27 for 7.1 Ab & 24 for TYN) tumours was evaluated and compared. Significant differences between B and M occurred in terms of the highest intensity (P = 0.0001, Fisher's exact test), the % of area stained (P< 0.040-0.042, Mann–Whitney test) and final score (% area x · intensity; P<0.003-0.027, Mann–Whitney test) for 7.1 Ab and TYN respectively.

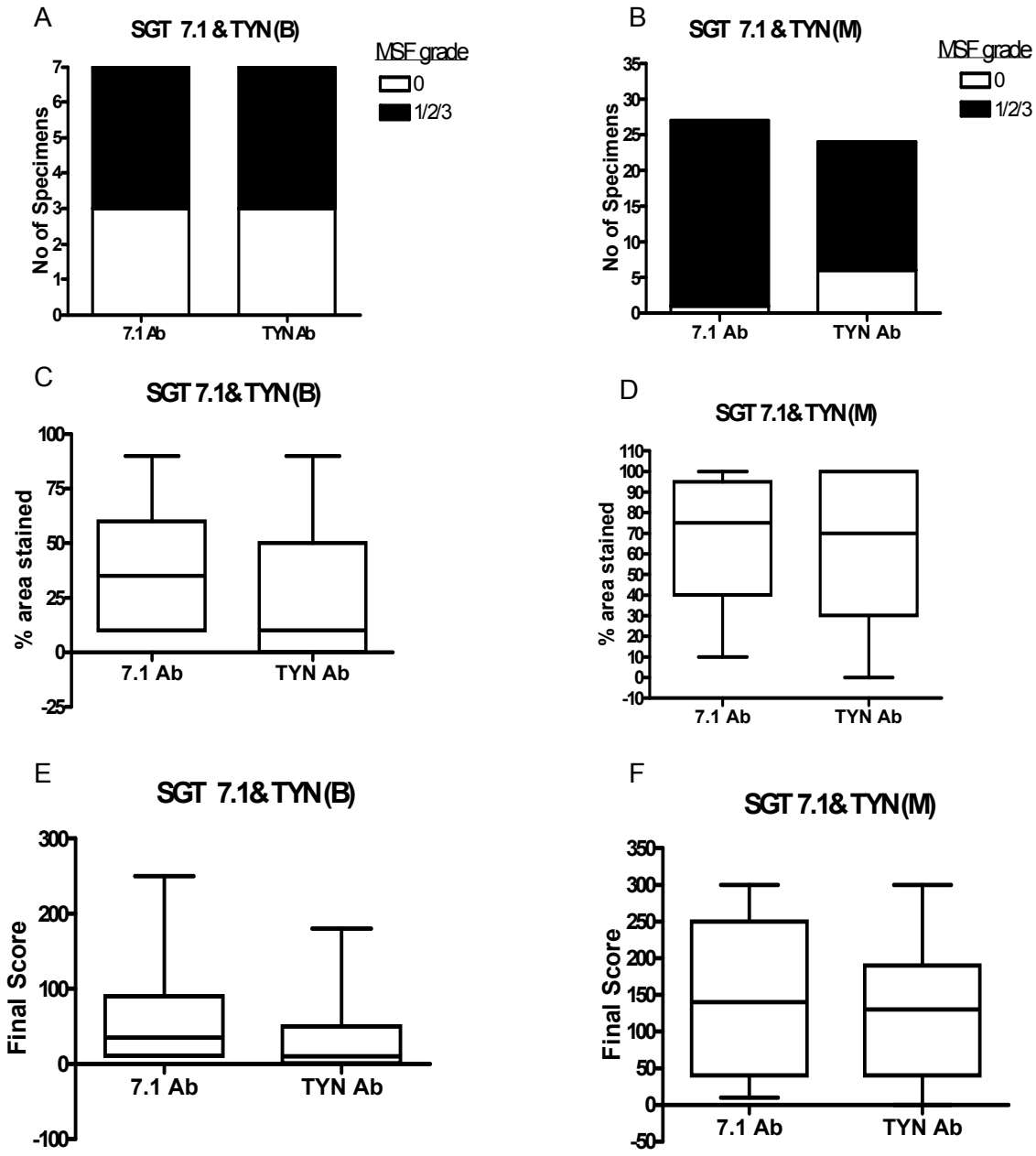


Figure 5.6: Comarison between two MSF antibodies (7.1 and TYN).
MSF expression in the epithelium of benign (B; n = 7) and malignant (M; n = 27 for 7.1 Ab & 24 for TYN) tumours was evaluated and compared. No significant differences between the two antibodies occurred in terms of the highest intensity regarding benign v benign (A) and malignant v malignant (B) (P=1-0.517 Fisher's exact test) respectively. similarly, no significant differences between the two antibodies occurred in terms of the % of area stained regarding B v B (C) and M v M (D) (P = 0.317-0.664, Mann–Whitney test) and final score (E, F) (% area x intensity; P =0.223-0. 0.243, Mann–Whitney test) respectively.

5.5 Discussion

The present study revealed that (i) significantly higher levels of total MSF and MSF-aa were detected in malignant than in benign salivary gland tumours, (ii) MSF staining was also apparent in some histologically normal salivary gland tissue, although this was significantly less than in either benign or malignant tumours. The expression of MSF in NSG showed the same pattern irrespective of whether the NSG was adjacent to SGT or OSCC. The difference in MSF expression between histologically normal tissue (adjacent to tumours) and benign tumours suggests that up-regulation of MSF expression may be a significant feature of early salivary gland tumour inception and/or progression. Furthermore, the observed higher expression of MSF by malignant tumours may prove to be a useful marker of later stage disease progression. Schor *et al,* (2003) previously reported that both MSF protein and mRNA are expressed by the same cell types in foetal skin and breast tissues. As is the case with breast cancers (Schor *et al.,* 2003; Schor and Schor, 2010). Some MSF expression was associated with the ductal epithelium of histologically normal salivary gland tissue adjacent to malignant tumours. It is important to note that normal salivary gland tissue from healthy adults was not available for examination in this study. This may be of significance in light of previous observations that MSF is expressed by fibroblasts obtained from histologically normal breast adjacent to mammary carcinomas, but not by normal breast tissue from healthy adults (i.e. reduction mammoplasty biopsies) (Schor *et al.,* 1994). It is therefore possible that MSF expression by the normal appearing salivary gland tissue represents a 'functional aberration' reflecting the proximal location of a malignant tumour and/or the result of a 'field cancerisation' effect resulting from previous exposure to a carcinogenic agent (Yoshino *et al.,* 2007, Slaughter *et al.,* 1953). In the case of the stromal vasculature, it is of interest that MSF was not detected in the blood vessels of the normal salivary gland, whereas a benign tumour derived from the vascular smooth muscle cells (angiomyoma) showed MSF staining. The complex histo-cytological structure of the salivary glands and their tumours leads to diagnostic difficulties.

5.6 Conclusion

Data presented here provide an initial indication that the expression of MSF isoforms expressions is up-regulated in both the epithelial and stromal cell compartments of malignant salivary gland tumours. This study provides a rational platform for subsequent more extensive investigation of the possible diagnostic and prognostic significance of MSF expression in this currently difficult to manage patient group. It also suggests that developing means to inhibit MSF expression and / or functionality may provide novel therapeutic strategies to improve the management of patients with salivary gland tumours.

5.7 Further studies

- A more detailed cytological study will be required to ascertain the possible relationship between MSF expression and the histological characteristics of the tumours.
- It would be of value to increase the numbers of benign tumour specimens to improve the statistical viability of the study.
- To assess the presence of MSF mRNA in salivary gland tumours by using *in situ* hybridization.

6. Chapter six: Identification of MSF Receptors

6.1 Introduction

Cell–cell and cell–matrix interactions play important roles in tumour metastasis and angiogenesis. One of the most important classes of receptors involved in these processes are the integrins. Integrins are a group of extracellular matrix receptors made up of an alpha and a beta chain, each chain being approximately 130-210kDa (alpha chain) and 95-130kDa (beta chain) in molecular weight. There are at least 14-18 different α subunits and 8 different β subunits, forming more than 20 heterodimers, many with numerous ECM ligands (Hynes, 1992; 2002). These proteins play an important role in the regulation of cell proliferation, growth, differentiation and migration of cells (Geiger *et al*., 2001 and Danen, 2005). In addition to their role in the maintenance of tissue integrity (Thomas and Speight, 2001), integrins transduce messages through various signalling pathways and influence proliferation and apoptosis of tumour cells, as well as of activated endothelial cells.

Specific amino acid sequences within ECM macromolecules, for example RGD, associate with the extracellular domain of integrin receptors. Integrins do not exhibit enzyme activity and require accessory proteins for signalling (Juliano and Haskill, 1993; Juliano, 1994; Clark and Brugge, 1995). Activation of integrins results in integrin clustering and assembly of focal adhesion complexes, linking the ECM to cytoskeletal components. Mueller *et al.,* (1989) demonstrated that fibronectin-coated beads induced organisation of cytoskeletal components on the inner membrane surface. Competitive inhibition of integrin binding using specific amino acid motifs, such as GRGDS for $\alpha_5\beta_1$ integrin, caused dissolution of cytoskeletal components from matrix focal adhesions (Stickel and Wang, 1988). The cytoskeletal molecules in focal adhesion contacts include α-actinin, talin, vinculin, paxillin and tensin. The cytoplasmic domain of the integrin β subunit interacts directly with α-actinin and talin. Mutations in the β_1 subunit result in reduced cell spreading and motility (Balzac *et al.,* 1994). Furthermore, whilst the β_1 and β_5 subunit support cell adhesion equally, β_5 showed less recruitment into focal adhesions and was associated with migration of fibronectin (Pasqualini and Hemler, 1994).

Integrin αvβ3

Integrin αvβ3 is one of the most prominent members of these receptor classes. It has been demonstrated that αvβ3 is an important receptor affecting tumour growth, local invasiveness, and metastatic potential (Hood and Cheresh, 2002). This integrin is expressed on various malignant tumours and mediates adhesion of tumour cells on a variety of extracellular matrix proteins, allowing these cells to migrate during invasion and extravasations (Felding-Habermann, 2003).

The integrin αvβ3 is also highly expressed on activated endothelial cells during angiogenesis (Brooks, 1994). In contrast, expression of αvβ3 is weak in resting endothelial cells and most normal organ systems (Brooks *et al.,* 1994). On activated endothelial cells, the receptor mediates migration through the basement membrane during formation of the new vessel, which is essential to provide sufficient nutrient supply for the growing tumour. Inhibition of the αvβ3-mediated cell–matrix interaction has been found to induce apoptosis.

Erdreich-Epstein *et al*, (2005) reported that integrin αvβ3-mediated adhesion to the extracellular matrix (ECM) and it is essential for endothelial cell growth and survival, while αvβ3 antagonism may induce endothelial apoptosis during angiogenesis.

Binding of any matrix macromolecule to the receptor elicits a chain of events including integrin clustering and consequent integrin-mediated intracellular signal transduction. After binding, integrins (which have no intrinsic enzymatic or kinase activities) activate complex signaling pathways by combining with kinases and adaptor proteins in focal adhesion complexes (Giancotti and Tarone, 2003). The complexes known as specialised adhesive structures, are composed of integrins, protein kinases – such as focal adhesion kinase (FAK) and the non-receptor tyrosine kinase receptor Src – adaptor protein such as Shc, signaling intermediates such as Rho family GTPases, actin-binding cytoskeletal proteins (such as talin, α-actinin, paxillin, tensin and vinculin), and other signaling proteins (Lo, 2006; Mitra, and Schlaepfer, 2006).

6.2 Aims

The objective of this study was to identify the receptors responsible for the motogenic activity of MSF and to determine whether the motogenic activity of MSF on various types of cells (oral tumour, salivary gland tumour and human endothelial cell lines) is mediated by αvβ3 receptor.

6.3 Materials and Methods:

Recombinant proteins: Recombinant αvβ3 (R&D Systems), rhMSF+aa and rhMSF-aa (prepared in house)

Antibodies:
PrimaryAb:

- Mab VSI 2.1.
- RpVSI, 2μg/ml.
- Anti- human integrin αVβ3 antibody Mouse.
- Anti- human integrin αVlCD 51 antibody Goat AF 1219.
- Anti-human Integrin β3/CD61 Monoclonal Antibody, MAB2266.
- TYN 1.1 (batch 3). Mouse monoclonal antibody to MSF- aa.

Secondary Ab:

- Goat anti-rabbit conjugated with horse radish peroxidase (GAR-HRP), (DAKO)
- Rabbit anti-mouse conjugated with horse radish peroxidase (RAM-HRP), (DAKO).
- Rabbit anti-goat conjugated with horse radish peroxidase (RAG-HRP), (DAKO)

Cells:
Different types of cell lines have been used in this study as in described in (Table 2.2) Chapter two Materials and Methods.

Affinity chromatography: Three affinity chromatography columns (rhMSF+aa, thMSF-aa and BSA) were prepared as described in Chapter two Materials and Methods.

Membrane proteins were extracted from pellets of cultured cells using the Mem- PER Eukaryotic Membrane Protein Extraction Reagent Kit from Pierce as described in Chapter two (Materials and Methods). In addition to the transmembrane migration assay as outlined also in Chapter two (Materials and Methods).

6.4 Results

6.4.1 Effects of anti- αvβ3 antibody on the MSF bioactivity Inhibition of MSF bioactivity by anti- αvβ3 antibody

The integrin αvβ3 integrin has been reported to mediate MSF activity on fibroblasts (Schor *et al.,* 1999; Ellis *et al.,* 2010). In this study the effects of neutralising antibody to this integrin on MSF-stimulated migration, on different target cells was investigated. Data presented in Fig 6.1, 6.2 and 6.3 indicate that stimulation of migration by MSF on HSG, TYS, and Endo 742 cells was abrogated by the addition of αvβ3 neutralising antibody. These data show that anti-αvβ3 antibody on its own did not affect the migration of these cells. However, when added together with rhMSF (100pg/ml), the chemotactic effects of MSF at 100pg/ml were completely neutralised by anti-αvβ3 at 1μg/ml concentration, suggesting that the motogenic activity of MSF is mediated by αvβ3 integrin.

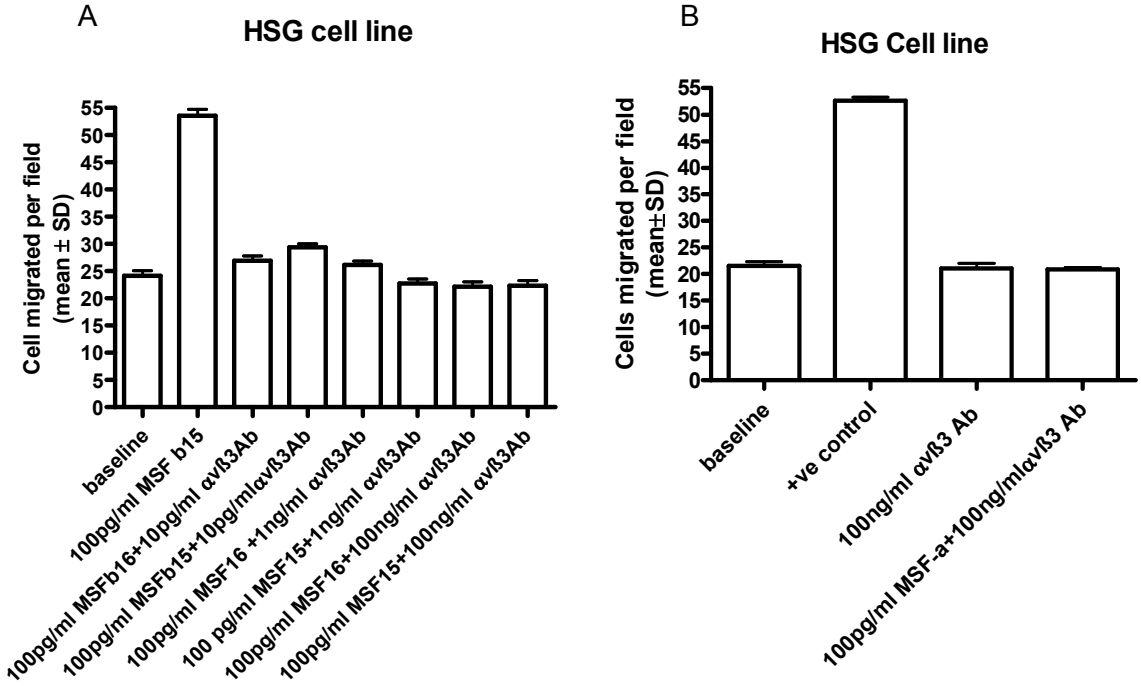


Figure 6.1: Effect of anti-alpha v beta 3 antibody on the motogenic activity of rhMSF on the HSG cells in the transmembrane assay.
Serum-free- MEM containing 2μg/ml BSA (baseline) was used as a negative control and two batches (15 & 16) of rhMSF+aa, 100pg/ml rhMSF-aa as a positive controls. A significant inhibitory effect of anti- αvβ3 on the motogenic activity of rhMSF on the migration of the HSG cell line was shown when compared to the negative control (Bonferroni's test).

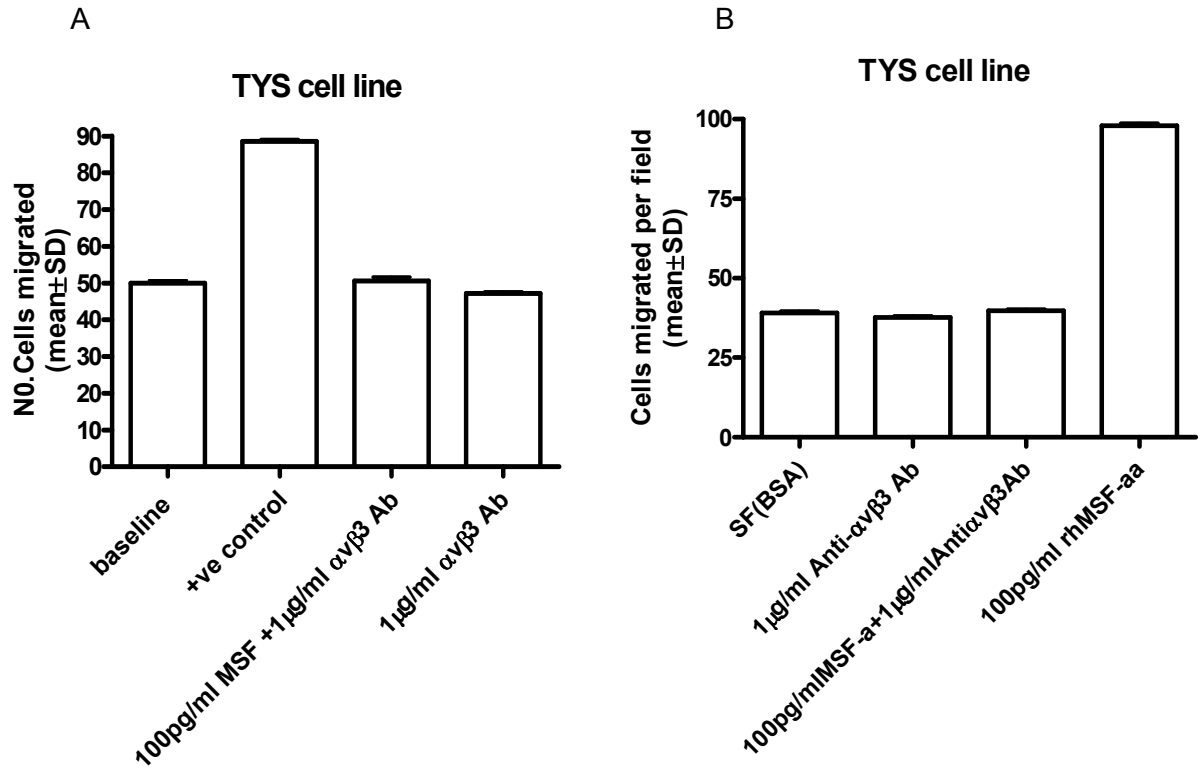


Figure 6.2: Effect of anti-alpha v beta 3 antibody on the motogenic activity of rhMSF on the TYS in the transmembrane migration assay.

Serum-free- MEM containing 2µg/ml BSA (baseline) was used as a negative control and 100pg/ml of rhMSF+aa & rhMSF-aa as a positive controls. A significant inhibitory effect of anti-αvβ3 on the motogenic activity of rhMSF on the migration of the TYS cell line was shown in comparison to the negative control (Bonferroni's test).

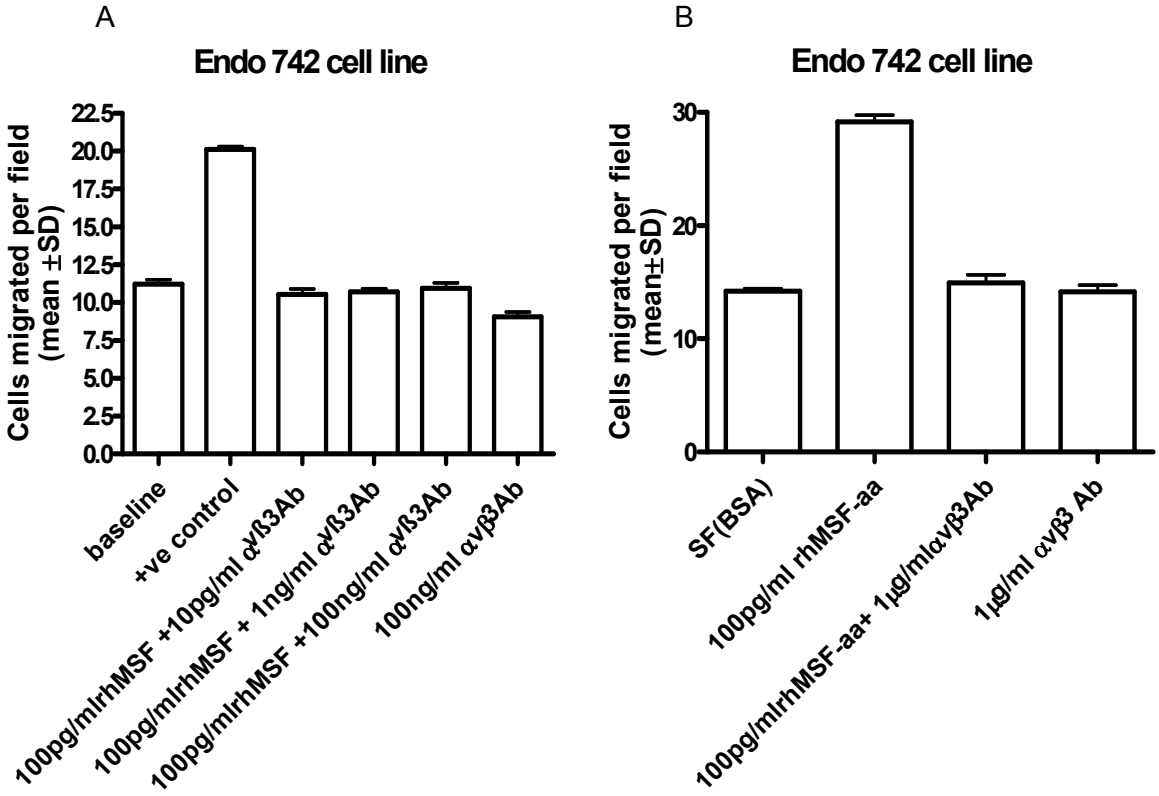


Figure 6.3: Effect of anti-alpha v beta 3 antibody on the motogenic activity of rhMSF on the Endo 742 cells in the transmembrane migration assay.

Serum-free- MEM containing 2µg/ml BSA (baseline) was used as a negative control and 100pg/ml rhMSF+aa (A) and rhMSF-aa (B) as a positive controls. All concentrations of anti-αvβ3 antibody inhibited the motogenic activity of rhMSF compared to the negative control (Bonferroni's P>0.05), furthermore there was no significant effect of anti- αvβ3(alone) on the migration of the Endo 742 cells in comparison to the negative control (Bonferroni's test).

The effect of anti-αvβ3 antibody on the bioactivities of MSF 3, 5m and 7, 9m mutants on the migration of Endo 742 cells were tested to determine the possible binding site of αvβ3 integrin with MSF. Data presented in (Fig 6.4) indicate that anti-αvβ3 antibody neutralised the motogenic activity of all mutants comparing to negative control (Bonferroni's test).

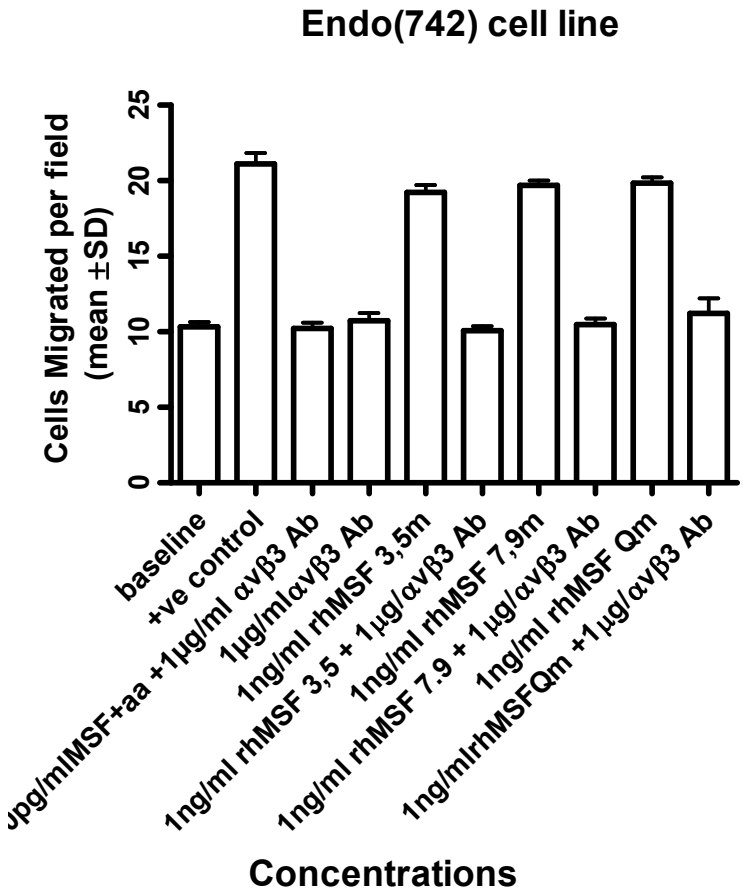


Figure 6.4: Effect of anti-alphav beta 3 antibody on the motogenic activity of MSF 3,5m, 7,9m and Qm on the Endo 742 cells in the transmembrane migration assay.
Serum-free- MEM containing 2µg/ml BSA (baseline) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. Anti- αvβ3 antibody inhibited the motogenic activity of all MSF mutants in comparison to the negative control (Bonferroni's test), all these mutants alone stimulate the migration of Endo 742 cells in comparison to the negative control (Bonferroni's test).

After TYS-CM affinity column chromatography with RpVSI MSF identification antibodies, fractions containing migration stimulating activity was analysed for the presence of MSF using the transmembrane assay and anti αvβ3 on different target cells. Anti αvβ3 did not affect the control or baseline migration of TYS cells when tested on its own, but in combination with the eluted fraction purified with RpVSI, it effectively reduced the cells migrated from values comparable to those of positive controls(100pg/ml rhMSF+aa) to baseline levels (Fig 6.5).

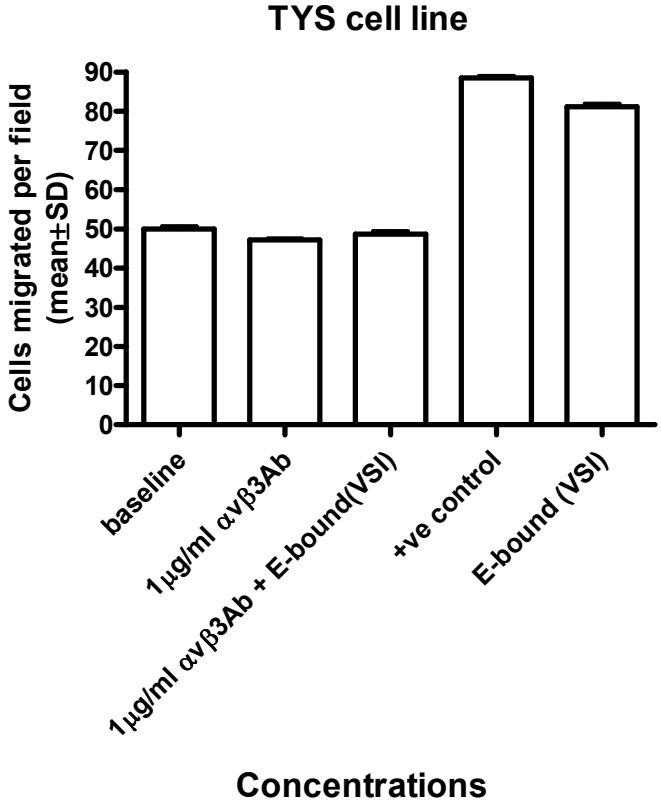


Figure 6.5: Effect of anti-alpha v beta 3 antibody on the motogenic activity of TYS-CM after characterisation by RpVSI affinity chromatography on the TYS cells.
Serum-free- MEM containing 2µg/ml BSA (baseline) was used as a negative control and 100pg/ml rhMSF+aa as a positive control. The motogenic activity of the eluted sample (MSF) was abrogated by the presence of anti-αvβ3 antibody in the migration assay. A significant effect of the eluted sample (MSF) on the migration of TYS cells was shown, in comparison to the negative control (Bonferroni's $P<0.05$).

6.4.2 Recombinant αvβ3 binding with rhMSF

In two preliminary experiments polystyrene 96-well microplates (Costar) were coated overnight with rhMSF, rhαvβ3 and cellular fibronectin (cFn). The second protein (rhMSF and rhαvβ3) was then applied to the plate diluted in PBS, the indirect ELISA method as described in Materials and Methods Chapter two was then followed. Data presented in (Table 6.1) indicated a direct association between rhαvβ3 and rhMSF.

Table 6.1: Summary of intial results; Binding between recombinant alph v beta 3 and rhMSF in indirect ELISA

	Basic ELISA				
Exp No	**Coating Protein (500ng/ml)**	**Second Protein (500ng/ml)**	**First Ab (1 or 10µg/ml)**	**OD 450-570nm mean of 2 wells**	**Comment**
1	αvβ3	MSF	Mab VSI 2.1 Anti-MSF 500ng/ml	0.272	Positive binding
	MSF	αvβ3	Mab3050 Anti-αvβ3 500ng/ml	0.186	Positive binding
	cFn	αvβ3	Mab3050 Anti-αvβ3 500ng/ml	0.275	Positive binding (positive control)
	MSF	none	Mab3050 Anti-αvβ3 500ng/ml	0.058	No binding (Negative control)
	CB	none	Mab3050 Anti-αvβ3 500ng/ml	0.028	No binding (Negative control)
2	αvβ3	MSF	RpVSI 10µg/ml Anti-MSF	0.479	Positive binding
	MSF	αvβ3	Anti αVlCD 51, 1µg/ml	0.808	Positive binding
	cFn	αvβ3	Anti αVlCD 51, 1µg/ml	0.774	Positive binding (positive control)
	αvβ3	none	RpVSI 10µg/ml Anti-MSF	0.038	No binding (Negative control)

cFn= cellular fibronectin (+ve control), CB= coated buffer

6.4.3 Binding of integrin αvβ3 with both MSF isoforms

Binding of integrin αvβ3 with rhMSF+aa, rhMSF-aa and BSA affinity chromatography columns was carried out as described in section (2.3.3) of Materials and Methods in Chapter two. The resultant fractions were assayed for αvβ3 concentration by indirect MSD ELISA, the plate was coated overnight with; rhαvβ3 at (0, 0.4, 2, 10, 50ng/ml) as a standard curve and eluted fractions from three affinity columns, using specific anti- αv antibody (Anti-human Integrin αV/CD51, AF 1219 antibody), and anti-β3 (Anti-human Integrin β3/CD61 Monoclonal Antibody, MAB2266). Data presented in (Table 6.2) (Fig 6.7 and 6.8A) indicate a direct association between αv, β3 subunits and both MSF isoforms, whilst there was no effect with BSA (negative control), in addition the concentration of αv and β3 in eluted fractions was measured by using linear regressions statistical test. Fig 6.6 showed standard curve of rhαvβ3 with both anti-αv and anti-β3 antibodies.

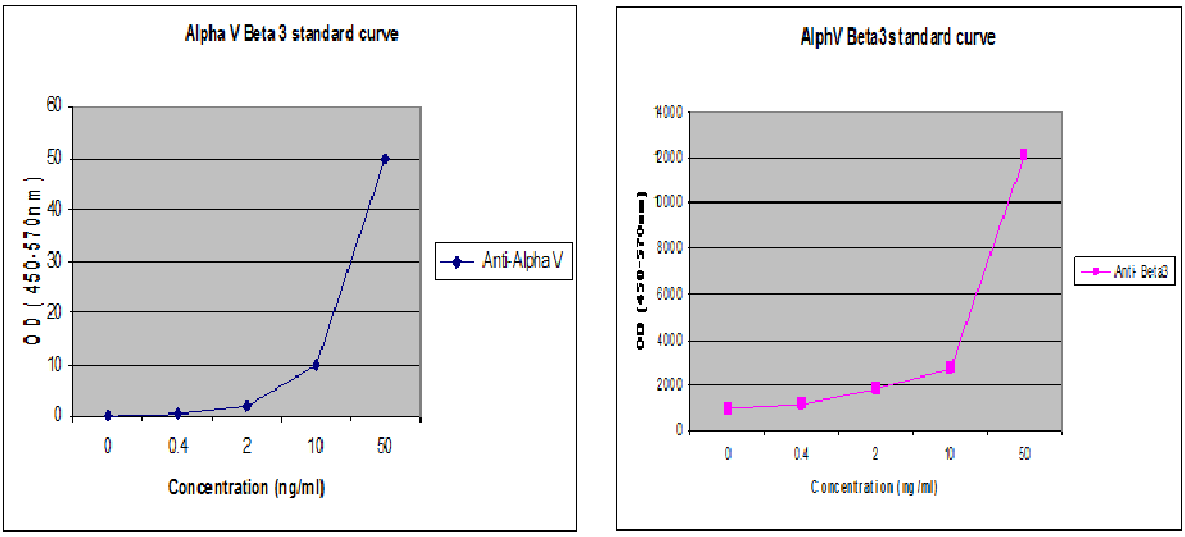


Figure 6.6: Indirect MSD ELISA standard curve of recombinant alpha v beta 3 using anti-alpha v and anti-beta 3 antibodies.

Table 6.2: Indirect MSD ELISA summary of eluted recombinant alpha v beta 3 across three affinity chromatography columns

antibody	Sample eluted from	OD mean value (counts)	Equivalent to Recombinant protein standard curve (ng/ml) Linear regression test	Comments
Anti- human integrin αVlCD 51 antibody AF 1219	MSF-aa column	3381±26.2	2.224ng/ml	Detectable
	MSF+aa column	6928.5± 38.8	4.834ng/ml	Detectable
	BSA column	86± 8.4	-5.876	undetectable
Mouse Anti-human integrin beta 3 (CD61) antibody	MSF-aa column	1247±141	1.039ng/ml	Detectable
	MSF+aa column	29512±735.3	8.820ng/ml	Detectable
	BSA column	24± 2.8	- 4.898	undetectable

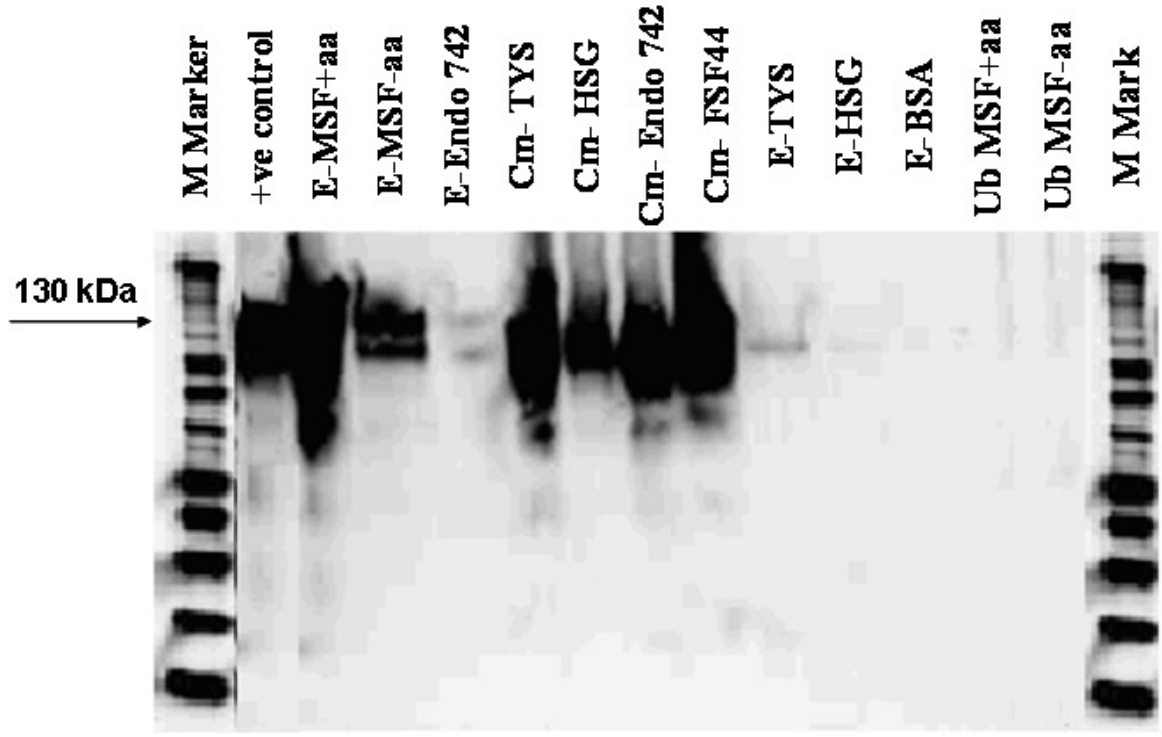


Figure 6.7: Binding and identification of integrin alpha v beta 3 using anti-alpha v antibody.
Recombinant αvβ3 (2µg/ml) was incubated with three chromatography affinity columns (rhMSF+aa, rhMSF-aa and BSA) as described in Materials and Methods in Chapter two. The eluted αvβ3 was fractionated by 7.5 % SDS PAGE under reducing conditions and immunoblotted with the commercial anti- αv. A clear band was seen at approximately 130 kDa, confirming the binding of αv subunit with both MSF isoforms (lane 3, 4). Whilst no band was seen with the sample eluted from the BSA column (lane 12). αvβ3 was identified in cell membrane proteins that had been extracted from four cell lines (TYS, HSG, Endo 742 and FSF44) (line 6-9), and also in the eluted fractions of TYS, HSG, Endo 742 (lanes 5, 10 and 11) after affinity chromatography following the same methods as described in Materials and Methods. The data indicate the presence of αv subunit in these cell membranes and in the eluted fractions that binding with total MSF. (M=magic marker, +ve control= recombinant αvβ3, E= eluted αvβ3 across affinity column, Cm=cell membrane, Ub= unbound fraction)

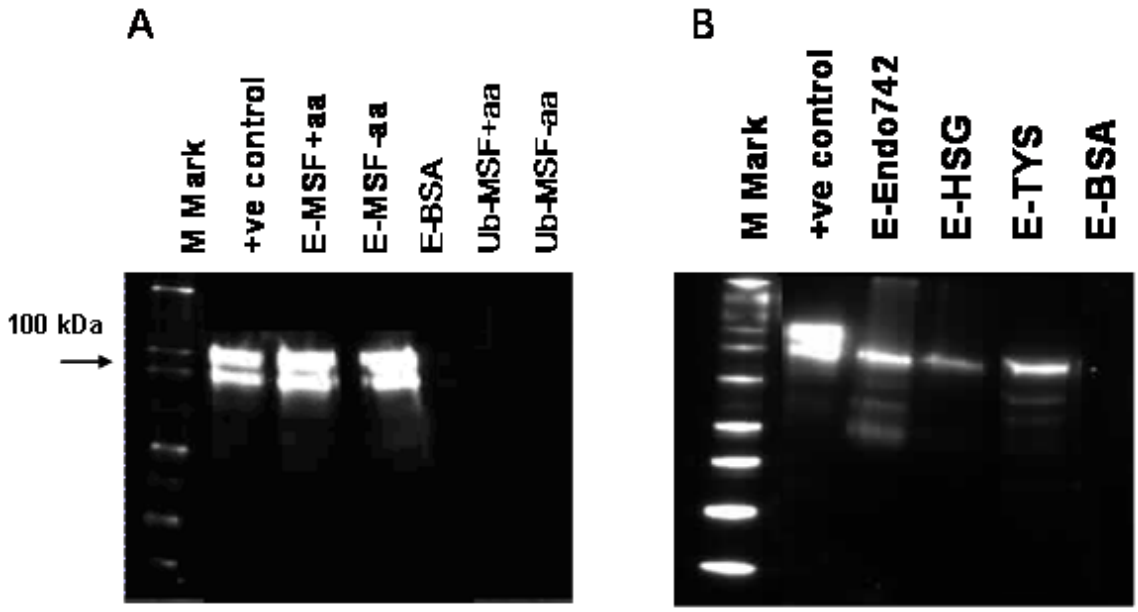


Figure 6.8: Binding and identification of integrin alpha v beta 3 using anti-beta 3 antibody.
(A) Recombinant αvβ3 (2µg/ml) was incubated with three chromatography affinity columns (rhMSF+aa, thMSF-aa and BSA) as described in Materials and Methods in Chapter two. The bound and subsequently eluted αvβ3 was fractionated by 7.5 % SDS PAGE under reducing conditions and immunoblotted with the commercial anti-β3. A clear band was seen at approximately 100 kDa, confirming the binding of β3 subunit with both MSF isoforms (lane 3, 4). Whilst no band with BSA column and unbound fractions (lanes 5, 6 and 7). (B) αvβ3 was identified in cell membrane proteins that had been extracted from (TYS, HSG, and Endo 742) cell lines after affinity purification against rhMSF (lanes 3, 4 and 5), following the same methods as described in Materials and Methods. The data indicate the binding of β3 subunit in the eluted fractions of these cell membranes with total MSF, but not with BSA (lane 6).

6.4.4 Identification of integrin αvβ3 in cell membrane extracts

Integrin αvβ3 was identified in samples of cell membrane proteins that had been extracted from three cell lines (TYS, HSG and Endo 742) using two chromatography affinity columns (rhMSF+aa and BSA as a negative control) following the same methods of RpVSI affinity chromatography as described in section (2.3.3) in Materials and Methods Chapter two. The eluted fractions for each sample were pooled after affinity chromatography and concentrated by freeze drying. Integrin αvβ3 was identified in the fractions by using indirect MSD ELISA and Western blot (Fig 6.7 and 6.8B) and Mass spectroscopy. Data presented in (Table 6.3) indicate that the integrin subunits αv and β3 are present in the cell membrane extracts and that both MSF isoforms bind to this receptor.

Table 6.3: Indirect MSD ELISA summary of eluted cell membrane proteins across three affinity chromatography columns

sample	antibody	Sample eluted from	OD mean value	Equivalent to Recombinant protein standard curve (ng/ml) linear regression test	Comments
Endo 742	Anti- human integrin αVlCD 51 antibody AF 1219	MSF+aa column	3319.5±6.3	2.188ng/ml	Detectable
		BSA column	67±23	0.33pg/ml	Undetectable
	Mouse Anti-human integrin beta 3 (CD61) antibody	MSF+aa column	13288±42.09	42.815ng/ml	Detectable
		BSA column	-552±13	-19.3	Undetectable
HSG	Anti- human integrin αVlCD 51 antibody AF 1219	MSF+aa column	1113.5±7.07	1.762ng/ml	Detectable
	Mouse Anti-human integrin beta 3 (CD61)antibody	MSF+aa column	4140±24.9	1.820ng/ml	Detectable
TYS	Anti- human integrin αVlCD 51 antibody AF 1219	MSF+aa column	5737 ± 79.4	3.565ng/ml	Detectable
	Mouse Anti-human integrin beta 3 (CD61) antibody	MSF+aa column	9436.5±30.9	2.695ng/ml	Detectable

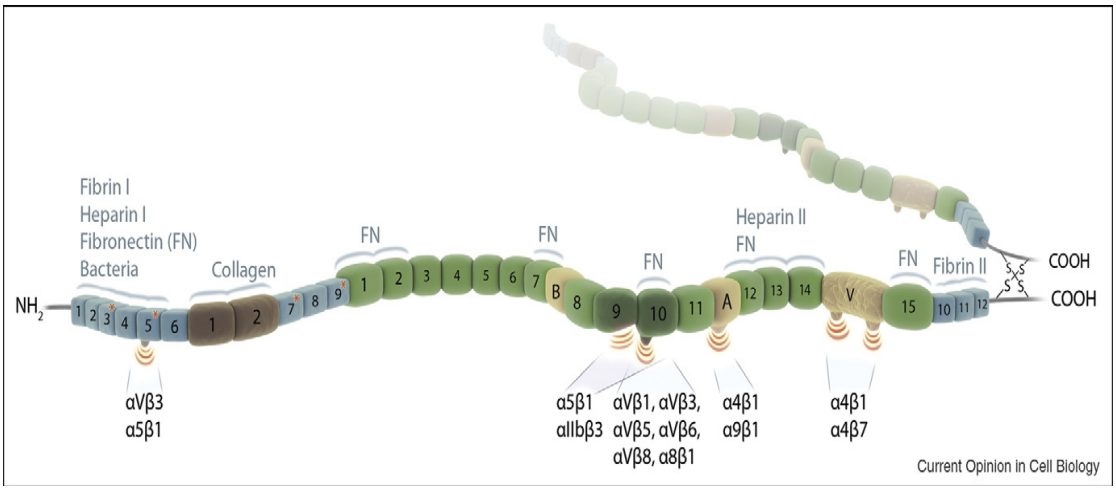


Figure 6.9: Schematic description of the modular structure of fibronectin.

Fn consists of three different modules (type I, blue; type II, brown; type III, green). The alternatively spliced extradomains B, A, and variable region (V) are shown in ochre. The dimer forms via two disulfide bonds at the C-terminus. Integrin binding sites are indicated. The N-terminal type I domains marked with a red asterisk harbour an IGD motif. Binding domains for Fn, collagen, fibrin, heparin and bacteria are indicated. Besides providing a high affinity binding site for αvβ3, the isoDGR motif in the fifth type I repeat also mediates low affinity binding to α5β1. (Adapted from Leiss *et al.,* 2008)

6.5 Discussion

Integrins are a family of heterodimeric, cation–dependent transmembrane glycoproteins that mediate cell adhesion and are involved in many cells signalling pathways (Hynes, 1992). The majority of integrin ligands are either ECM molecules (e.g. fibronectin and vitronectin) or cell surface molecules of the immunoglobulin superfamily (e.g. intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)). Some ligands, like fibronectin bind to several integrins including- α5β1, αvβ1, α4β1, αIIβ3, αvβ6 (Fig 6.9). Some integrins, like αvβ3 binds to several ligands including fibronectin, fibrinogen, bone sialoprotein, laminin, thrombospondin, vitronectin, and van Willebrand factor (Humphries, 1990; Felding-Habermann and Cheresh, 1993; Newham and Humphries, 1996; Horton, 1997; Boettiger *et al.,* 2001a and 2001b). The enormous and difficult network of receptor–ligand complexes permits integrins to contribute to a variety of cellular processes, such as cellular adhesion, migration, proliferation, differentiation, and survival (Geiger *et al*., 2001; Howe *et al*., 1998).

Integrins may play an important role in tumour cell invasion and metastasis in OSCC, such as αvβ3, αvβ6, α5β1 which are not expressed in healthy oral epithelia but are upregulated during carcinogenesis, angiogenesis or wound healing. However, invasion can be reduced dramatically in OSSC cell lines using anti-αv or anti- αvβ6 antibodies (Brooks *et al.,* 1994; Eliceiri and Cheresh, 2000; Thomas *et al.,* 2001 and Thomas *et al.,* 2006)

The aim of this study was to identify the receptors responsible for the motogenic activity of MSF and to verify that the motogenic activity of MSF in various types of cells (oral tumour, salivary gland tumour and human endothelial cell lines) is mediated by αvβ3 receptor.

Data presented in this study indicates a direct association between αvβ3 integrin and rhMSF protein. In addition to that, the direct association between αv, β3 subunits (eluted fractions) and both MSF isoforms after affinity chromatograph. MSF can bind to and might be able to signal via integrin αvβ3.These result reflect that the possible mechanism whereby functional neutralising antibody to αvβ3 abolished the motogenic effects of MSF on different target cells (TYS, HSG and Endo742), our results are in agreement with Schor *et al,* (1999) and Ellis et al, (2010) who reported that fibroblast cell migration is activated by IGD and GBD of fibronectin but was blocked by a neutralising antibody to integrin αvβ3. They also showed that migration

is mediated by a signal transduction cascade involving the tyrosine phosphorylation of FAK. This may reflect the importance of integrin αvβ3 in IGD stimulated migration.

Brooks et al, (1995) and Rathinam and Alahari, (2010) reported that in tumour cells, the αvβ3 integrin promotes growth and invasion, whereas its inhibition results in anoikis [apoptosis that is induced by inadequate or inappropriate cell-matrix interactions. It is involved in a wide diversity of tissue-homeostatic, developmental and oncogenic processes (Frisch and Screaton, 2001)] and tumour regression as an anti-angiogenic agent for the treatment of cancer.

Thomas *et al,* (2001) showed in their study that αvβ6 increased matrix metalloproteinase production, stimulated cell migration and increased invasion of oral tumour cells. Schor *et al,* (1999) indicated that response of fibroblasts to MSF/IGD requires maintenance of integrin αvβ3 functionality and is mediated, at least in part, by the PI-3 kinase signal transduction pathway. Ellis *et al,* (2010) found that Migration stimulating activity of IGD containing molecules on fibroblasts was blocked by neutralising antibodies to integrin αvβ3 and is mediated by a signal transduction cascade involving the tyrosine phosphorylation of Focal adhesion kinase (FAK).

Integrins are known to act via RGD containing peptide motifs and have been reported to cause the phosphorylation of integrin associated proteins such as FAK. The RGD loop has been recognised as key sequence for integrin αvβ3 (Xiong *et al.,* 2002) but changing IGD to RGD abolished the motgenic activity of fibroblast cells which may be due to inherent inflexibility of RGD in this circumstance (Millard *et al*., 2007).

Our results also indicate that the identification of integrin αvβ3 from cell membrane proteins of different target cells including; TYS, HSG and Endo 742 by using two MSF isoforms chromatography affinity columns reflect the bidirectional signaling mechanism of integrin αvβ3 in the cellular functions (migration) of these cells by the interaction with MSF ligand at the site at which αvβ3 binds MSF which might be IGD site rather than an RGD site which is not present in the MSF structure.

Takahashi *et al,* (2007) reported that the absence of a functional RGD motif in fibronectin did not compromise the assembly of Fn fibrils in mutant embryos or cells. A possible explanation suggested was that Fn contains another integrin binding site in its N-terminal region other than RGD sequence (Fig 6.9).

On the other hand, Takahashi *et al.,* (2007) reported that other sequences mimic RGD, these sequences are DGR, NGR of which MSF has three and RGF of which MSF has two. The N-terminus of fibronectin (and hence MSF) contains two NGR which are located in 5Fn1 and 7Fn1 modules (Di Matteo *et al.,* 2006). The conversion of NGR to isoDGR sequence through the deamidation and rearrangement of the asparagine residue could constitute a binding ligand for the αvβ3 integrin, which may regulate the protein functions (Curnis *et al*., 2006).

Leiss *et al,* (2008) reported that the integrin αvβ3 binding site was located at the N-terminus of the Fn polypeptide and included the N-terminal type I domains (Fn-I1–9 fragment; also called the 70 kDa fragment of Fn).

Shpiro et al, (2005) designed and synthesised the first IGD peptidomimetic. These mimetics are able to stimulate the migration of human skin fibroblasts into 3D collagen gels (Shpiro *et al.,* 2005 and Marquez, *et al* unpublished). Modelling of the active RGD binding region of the integrin αvβ3 showed that the peptido-mimetic IGD could be docked comfortably onto the integrin and shares some of the RGD binding site (Norman *et al,* unpublished; Shpiro *et al.,* 2005).

Data presented here suggest that the motogenic activity of MSF in various types of cells (oral tumour, salivary gland tumour and human endothelial cell lines) is mediated by αvβ3 receptor, and the potential integrin αvβ3 binding site was located at the IGD motifs.

MSF affinity chromatography and MALDI (MS/MS) analysis were used to analyse membrane protein extracts of the cell line TYS, unfortunately no new candidate receptors for MSF were identified by the initial study.

6.6 Further studies

- Recombinant MSF-fragments as well as recombinant integrins are important tools, which are now available to perform ELISA- or Surface Plasmon Resonance-based binding studies to precisely map binding sites.

- To corroborate the initial data suggesting that the effect of MSF upon cells is a direct result of MSF binding to αvβ3 by using Surface Plasmon Resonance (SPR).

7. Chapter seven: Discussion, Conclusions and Suggestions

7.1 Discussion

Fibronectin is a multifunctional glycoprotein; expressed fibronectin bioactivities are dependent upon functional domains that are present in both the native-full length molecule and in its fragments. Fibronectin fragments created by proteolysis exhibit many properties not seen in the full length molecule, for instance GBD functional domain, which is generated proteolytically, exhibits a potent motogenic activity that is cryptic in full-length fibronectin (Schor *et al.,* 1996). The main function of fibronectin is cell adhesion but it appears to have many cryptic activities (Clark *et al*., 1988; Hynes, 1990; Emod *et al.,* 1990 and Fukai *et al.,* 1993). MSF being a truncated form of fibronectin possesses different activities to full length fibronectin (Schor *et al*., 2003; Houard *et al.,* 2005).

MSF is a potent motogenic factor, able to stimulate the migration of fibroblasts, epithelial and endothelial cells. MSF also stimulates hyaluronic acid (HA) synthesis and angiogenesis. It is important to note that none of these bioactivities are manifest by all previously characterised full-length isoforms of fibronectin and are most likely a consequence of steric hindrance of their constitutive bioactive motifs (Schor *et al.,* 2003; Millard *et al.*, 2007; Vakonakis *et al.*, 2009). In the 3D collagen gel migration assay MSF stimulates fibroblast migration whereas fibronectin has no effect (Schor *et al.,* 2003; Schor *et al.,* 1990). A study carried out by Schor *et al.,* (2003) revealed that the motogenic activity of MSF on fibroblasts is totally dependent on the GBD, as the mutation of IGD sequences of 7Fn1 and 9Fn1 module of the GBD inhibited the migration stimulating activity of MSF. Ellis *et al,* (2010) found that IGD sequences in 3FnI and 5FnI have a cryptic motogenic activity present within MSF fragments. The bioactivities of MSF have been discussed in Chapter one, several studies have shown the effect of MSF on the proliferation and migration of fibroblast target cells, Schor *et al,* (1988) and Grey *et al,* (1989) found that MSF does not affect either proliferation or morphology of normal adult cells under any of the culture conditions (Fetal or breast cancer patient skin fibroblasts; CM examined). They reported that the ability of MSF to stimulate fibroblast migration is not dependent upon the initiation of cell proliferation.

7.1.1 MSF Role in tumour pathogenesis

MSF is expressed by the three principal cell types of foetal skin (i.e. keratinocytes, fibroblasts and microvascular endothelial cells), not expressed by these cells in the majority of adult skin, and persistently re-expressed in tumours by both the carcinoma and stromal cell populations. MSF bioactivity in tumours may be responsible for increased levels of migration and invasion. The stimulation of tumour cell migration, biosynthesis of HA and angiogenesis are the mechanisms through which the potent bioactivities of MSF contribute to tumour pathogenesis (Schor *et al.,* 2003). Foetal development and the maintenance of normal tissue structure and function in the adult are mediated by epithelial and stromal cells interactions. These interactions are mediated by various soluble molecules, matrix macromolecules and chemical signals. Disturbance in these interactions could contribute to the aetiology of various pathological conditions, like cancer.

The precise response of potential target cells to MSF is modulated by a complex hierarchy of control mechanisms, including (i) the precise nature of the extracellular matrix and (ii) the presence of soluble and insoluble inhibitory molecules. For example, MSF is a potent stimulator of fibroblast migration on a native type I collagen substratum, but is completely devoid of motogenic activity on denatured type I collagen (Schor *et al.,* 2003). Interestingly, the response of carcinoma cells, stromal fibroblasts and endothelial cells to MSF are differentially modulated by matrix molecules: i.e. a given matrix constituent may affect the behaviour of one cell type, but not another. In this regard, previous data suggest that the stimulation of adult fibroblast migration by MSF may be a secondary consequence of its primary effect upon the deposition of matrix macromolecules, in particular HA (Schor *et al*., 1989; Schor *et al.* unpublished data). However, this is not the case with endothelial or other types of cells, where MSF does not affect HA synthesis (unpublished data). TGF-β1, a well characterised cytokine, may act as either an activator or an inhibitor of MSF, depending on the nature of the extracellular matrix in contact with the cells (Ellis *et al.,* 1992; Schor *et al.,* 2010; unpublished data).

In the present study the immunostaining with two MSF antibodies (7.1 and TYN) indicated that approximately 98% of OSCC (n = 50), 93% of malignant SGT (n= 24) and 43% of benign SGT (n=7) overexpressed total MSF and MSF-aa in both epithelial and stromal compartments. Oral tumour (TYS) and salivary gland tumour (HSG) cell lines produced MSF and secreted bioactive MSF into their conditioned

medium. Exogenous rhMSF stimulated the migration of tumour cells through collagen-coated membranes. Conversely, cell migration by MSF producing tumour cells was effectively abolished by MSF-function-neutralising antibodies (PEPQ) and anti-αvβ3 antibody. Of the two inhibitory factors examined, IGFBP7 inhibited both MSF isoforms whereas NGAL inhibited only MSF+aa.

Taken together, previous observations and our results suggest that cellular response to MSF in tumours is not invariant, but dependent upon spatial and temporal changes in the expression of MSF inhibitors and matrix re-modelling which occur during tumour progression.

The possible detrimental effects of MSF on disease outcome is supported by my present data indicating that high MSF expression by the cells in the invasive tumour front (ITF) is associated with poor survival in patients with OSCC. Evidence supporting the suggested role for MSF in cancer pathogenesis has also been presented by Hu *et al,* (2009); using an unbiased proteomic screen these authors identified MSF as a critical angiogenic factor driving oesophageal cancer progression.

The present study investigated the possible amino acid active motif that modulated the MSF migration stimulation for FSF44, Endo 742, TYS and HSG cells. The results indicated that the HEEGH motif functions in addition to the bioactive IGD motifs in stimulating the migration of target endothelial cells: i.e. both the HEEGH and IGD motifs of MSF must be mutated in order to abolish the motogenic response of endothelial cells. In the case of TYS and HSG cells, a single mutation in either IGD or HEEGH motifs is sufficient to abolish the motogenic response.

Schor *et al,* (1999) reported that the presence of αvβ3 and its downstream signalling components (phosphatidylinositol 3 kinase and focal adhesion kinase) are required for IGD motogenic activity. Our data provide evidence that MSF was found to bind directly integrin αvβ3, this interaction may lead to localisation of MSF in a proteolytically active form on the cell surface. These findings might provide a molecular basis to explain that this cell-surface receptor may be the most important receptor amongst a complex of other integrin and non-integrin receptors that can regulate both cell migration and matrix degradation, thereby facilitating invasion. This supports work by Houard *et al,* (2005) who suggested that the localisation of MSF at the cell surface may be necessary for the biological activity of MSF as a Fn-proteinase. Furthermore, Schor *et al,* (1999) reported that the IGD sequence resembles the integrin-binding sequence Arg-Gly-Asp (RGD), and it is possible that IGD

sequence targets MSF at the cell surface, thus initiating signal transduction pathways responsible for the migratory activity of MSF. Further studies are required to explain the exact mechanism of the IGD and HEEGH motifs for stimulating the migration of oral cancer cells.

MSF was over-expressed in malignant salivary gland tumours and oral sequamous cell carcinomas by both tumour cells and tumour-associated stromal cells. It is of particular interest that several sub-sets of stromal cells, including fibroblasts, microvascular endothelial cells and inflammatory cells, were positively stained, thus indicating the coordinated up-regulation of MSF expression during tumour progression. In this regard, it should be noted that MSF expression by stromal fibroblasts has recently been reported to be induced by epigenetic mechanisms regulated by the concerted signalling of TGF-β and the matrix (Kay *et al.,* 2005; Schor *et al.,* 2005; Schor and Schor, 2010). Considering the strong association between tobacco consumption and head and neck cancer incidence, it is of interest that the tobacco carcinogen benzo(a)pyrene has been shown to induce MSF expression by a bronchioloalveolar carcinoma cell line (Yoshino *et al.,* 2007).

7.2 Conclusions

- Two MSF isoforms (MSF+aa and MSF-aa) have been detected in tissue culture and in tissue sections. The general term MSF (or total MSF) is used here to refer to both isoforms. MSF+aa and MSF-aa induced the same migration-stimulating effect upon oral tumour cell lines (TYS and HSG) and normal stromal cells: skin fibroblasts (FSF44) and microvascular endothelial cells (Endo 742).

- Five bioactive motifs (4x IGD and 1x HEEGH) were found to be required for MSF bioactivity on TYS and HSG cells, whereas only one of these motifs was required for Endo 742 and two for FSF44.

- MSF+aa and MSF-aa differed in their interaction with the MSF-inhibitor NGAL. NGAL was shown to bind to and inhibit MSF+aa, but not MSF-aa. The bioactivity of MSF+aa and MSF-aa was inhibited by IGFBP7, MSF-function-neutralising antibody and antibody to the integrin αvβ3.

- Oral tumour cell lines TYS and HSG cells secreted bioactive MSF in culture. Therefore, MSF stimulates tumour cell migration in an autocrine and paracrine manner, modulated by the type of MSF isoform expressed and by the presence of NGAL and other possible inhibitors. MSF stimulated the migration of these cells.

- The integrin αvβ3 was identified in the cell membrane material bound to MSF, suggesting that αvβ3 is a receptor for MSF.

- In tissue sections, MSF was expressed by most OSCC and malignant SGT, being heterogeneously present in both carcinoma and stromal cells.

- Significantly higher levels of total MSF and MSF-aa were detected in malignant than in benign SGT. In OSCC, high MSF expression in the invasive tumour front was significantly associated with poor patient survival. MSF-aa was more informative than total MSF.

- Data presented here provide an initial indication that expression of MSF is up-regulated in both the epithelial and stromal cell compartments of malignant oral tumours. This study provides a rational platform for subsequent more extensive investigation of the possible diagnostic and prognostic significance of MSF in these tumours. It also suggests that developing means to inhibit MSF expression and／or functionality may provide novel therapeutic strategies to improve the management of patients with oral tumours.

7.3 Suggestions

- Study the expression of MSF in the normal oral mucosa and non-tumour lesions such as epithelial dysplastic tissue to provide a rational platform for subsequent more extensive investigation of the possible diagnostic significance of MSF expression in OSCC.

- To examine the role of MSF expression, in Kp or at the ITF, as a prognostic factor in larger number of patient samples.

- A more detailed cytological study will be required to ascertain the possible relationship between MSF expression and the histological characteristics of the salivary gland tumours.

- Increase the numbers of benign tumour specimens to study the diagnostic role of MSF expression in salivary gland tumour.

- Assessment the presence of MSF message in salivary gland tumours and OSCC by using in situ Hybridization.

- Using other types of tissue target cells for testing the migration effect of MSF.

- Investigate the role of other soluble molecules on the MSF bioactivity.

- Recombinant MSF-fragments as well as recombinant integrins are important tools, which are now available to perform ELISA- or Surface Plasmon Resonance-based binding studies to precisely map binding sites.

- To corroborate the initial data suggesting that the effect of MSF upon cells is a direct result of MSF binding to αvβ3 by using Surface Plasmon Resonance (SPR).

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- The study of the possible role of MSF on oro-facial development due to its oncofetal pattern of expression, and its possible role in facial deformities like cleft lip or palate.

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APPENDICES

Appendix 1: Boyden chamber migration assay of HSG cells and MSF

Exp 31: Code: LA18/05/09

Objective: To determine the inhibition effect of PEPQ 1.1 Ab on the migration of HSG.

Cells: salivary gland tumour cells, HSG
Cells: semi-confluent (85%), 3.6 x 10^6 cells/90mm dish
Cells have been grown on 90 mm dishes in 15% DCS-MEM- media
Last farmed 1x90mm to 2 x90mm, 1 day before the experiment on 17/05/09

Protien:

- **rhMSF +aa, Endotoxin free**, batch 3, of Conc. 10µg/ml diluted in 1ml of SF- BSA to final concentration of 100pg/ml and 100ng/ml.

Antibody:

- **PEPQ 1.1:** Stock concentration 61.4 µg/ml, Batch no.2, tested at final concentrations of 1µg/ml.

Upper chamber**:**Cells in SF-BSA (2 µg/ml);
4.5 x10^4cells/50µl/well (9 x 10^5 cells/ml)

Lower chamber**:** with SF-BSA (2 µg/ml)

SF-MEM (BSA)	100pg/ml MSF+aa	100ng/ml MSF+aa	SF-MEM (BSA)
SF-MEM (BSA) + 1µg/ml PEPQ1.1	100pg/ml MSF + 1µg/ml PEPQ1.1	100ng/ml MSF+aa + 1µg/ml PEPQ1.1	SF-MEM (BSA) + 1µg/ml PEPQ1.1

Filter used: 8µm pore filter used, immersed in collagen at 4° for overnight.
Native Collagen: 100 µg/ml used 1 times
Incubation period: 5 hours
Staining time: overnight
Negative control: Serum free- bovine serum albumin (BSA)

Result: Cells are counted 3 fields/well, and the mean of 6 wells per each variable using a microscope x 200(including eye piece magnification) under bright field conditions.

Conditions	Well	Migrated cell number			Mean/ well	SD/ well	Mean/6 well	SD/6 well	General mean	General SD	%
Serum free	1	30	33	29	30.667	2.0817	30.33333	0.843274			
	2	32	31	29	30.667	1.5275					
5 hr	3	29	30	30	29.667	0.5774					
	4	30	28	29	29	1			30.02778	1.039214	100%
d=-2	5	31	29	32	30.667	1.5275					
HSG	6	31	31	32	31.333	0.5774					
	7	29	28	30	29	1	29.72222	1.200309			
	8	31	30	30	30.333	0.5774					
	9	32	30	28	30	2					
2	10	30	29	28	29	1					
	11	29	28	28	28.333	0.5774					
	12	31	33	31	31.667	1.1547					
Serum free + PEPQ	1	29	30	30	29.667	0.5774	30.44444	1.772214			
	2	31	34	33	32.667	1.5275					
5 hr	3	28	28	27	27.667	0.5774					
	4	31	30	30	30.333	0.5774			30.36111	1.898741	101%
d=-2	5	32	34	30	32	2					
HSG	6	29	31	31	30.333	1.1547					
	7	28	27	29	28	1	30.27778	2.184965			
	8	32	31	31	31.333	0.5774					

	9	31	34	34	33	1.7321					
2	10	31	28	30	29.667	1.5275					
	11	27	27	29	27.667	1.1547					
	12	32	33	31	32	1					
100pg/ml	1	58	56	56	56.667	1.1547	58.33333	1.763834			
rhMSF+aa	2	57	56	57	56.667	0.5774					
5 hr	3	57	58	57	57.333	0.5774					
	4	60	59	57	58.667	1.5275			58.33333	1.763834	194.20%
	5	58	60	61	59.667	1.5275					
1	6	60	61	62	61	1					
100pg/ml+ PEPQ	1	33	32	28	31	2.6458	30.66667	3.040468			
rhMSF +aa	2	34	30	35	33	2.6458					101.90%
5 hr	3	24	25	26	25	1			30.66667	3.040468	
	4	32	30	28	30	2					
	5	31	35	34	33.333	2.0817					
1	6	31	31	33	31.667	1.1547					
100ng/ml	1	70	71	68	69.667	1.5275	71.16667	1.94079			
rhMSF +aa	2	70	72	72	71.333	1.1547					236.80%

5 hr	3	74	73	74	73.667	0.5774					
	4	74	73	73	73.333	0.5774			71.16667	1.94079	
d=-2	5	69	70	70	69.667	0.5774					
1	6	69	70	69	69.333	0.5774					
100ng/ml + PEPQ	1	29	31	33	31	2	31.16667	1.394433			
rhMSF+aa	2	28	32	31	30.333	2.0817					103.50%
5 hr	3	34	34	32	33.333	1.1547					
	4	31	32	34	32.333	1.5275			31.16667	1.394433	
d=-2	5	30	29	30	29.667	0.5774					
1	6	30	29	32	30.333	1.5275					

Table: effect of MSF +aa & PEPQ 1.1 Ab on the migration of HSG cells

	Mean	SD	% migration	Significance
				ANOVA & Bonferroni's (P<0.05)
SF-BSA 1+2	30.02	1.03	100	
SF-BSA+ 1µg/ml PEPQ	30.3	1.8	101	No
100pg/ml MSF+aa	58.3	1.7	194.2	Yes
100pg/ml MSF+aa+ 1µg/ml PEPQ	30.6	3.04	101.9	No
100ng/ml MSF+aa	71.1	1.9	236.8	yes
100ng/ml MSF+aa+ 1µg/ml PEPQ	31.1	1.3	103.5	No

Statistical analysis using One-way analysis of variance Bonferroni's test

Parameter	Value
Table Analyzed	
Data 1	
One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	657.1
R squared	0.9874

Bartlett's test for equal variances	
Bartlett's statistic (corrected)	8.855
P value	0.1150
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	11100	5	2219
Residual (within columns)	141.9	42	3.378
Total	11240	47	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
baseline vs SF(BSA) + 1mg/ml PEPQ	-0.3333	0.4443	P > 0.05	-2.669 to 2.002
baseline vs 100pg/ml rhMSF+aa	-28.31	30.80	P < 0.001	-31.17 to -25.45
baseline vs 100pg/ml MSF +aa +1mg/ml PEPQ	-0.6389	0.6952	P > 0.05	-3.499 to 2.221
baseline vs 100ng/ml MSF +aa	-41.14	44.77	P < 0.001	-44.00 to -38.28
baseline vs 100ng/ml MSF +aa + 1mg/ml PEPQ	-1.139	1.239	P > 0.05	-3.999 to 1.721
SF(BSA) + 1mg/ml PEPQ vs 100pg/ml rhMSF+aa	-27.97	30.44	P < 0.001	-30.83 to -25.11
SF(BSA) + 1mg/ml PEPQ vs 100pg/ml MSF +aa +1mg/ml PEPQ	-0.3056	0.3325	P > 0.05	-3.166 to 2.555
SF(BSA) + 1mg/ml PEPQ vs 100ng/ml MSF +aa	-40.81	44.40	P < 0.001	-43.67 to -37.95
SF(BSA) + 1mg/ml PEPQ vs 100ng/ml MSF +aa + 1mg/ml PEPQ	-0.8056	0.8766	P > 0.05	-3.666 to 2.055
100pg/ml rhMSF+aa vs 100pg/ml MSF +aa +1mg/ml PEPQ	27.67	26.07	P < 0.001	24.36 to 30.97
100pg/ml rhMSF+aa vs 100ng/ml MSF +aa	-12.83	12.09	P < 0.001	-16.14 to -9.531
100pg/ml rhMSF+aa vs 100ng/ml MSF +aa + 1mg/ml PEPQ	27.17	25.60	P < 0.001	23.86 to 30.47
100pg/ml MSF +aa +1mg/ml PEPQ vs 100ng/ml MSF +aa	-40.50	38.17	P < 0.001	-43.80 to -37.20
100pg/ml MSF +aa +1mg/ml PEPQ vs 100ng/ml MSF +aa + 1mg/ml PEPQ	-0.5000	0.4712	P > 0.05	-3.803 to 2.803
100ng/ml MSF +aa vs 100ng/ml MSF +aa + 1mg/ml PEPQ	40.00	37.70	P < 0.001	36.70 to 43.30

Appendix 2: Indirect MSD ELISA summary of eluted rhNGAL across three affinity chromatography columns

Concentration of NGAL in samples eluted from different affinity chromatography columns. The concentration was estimated by linear regression. The average OD of the buffer (PBS) was subtracted from the sample OD to obtain the OD result.

1. rh protein

- rhLipocalin Bach no 6 (Endotoxin free)30µg/ml & 10µg/ml and batch no 5, 5µg/ml at [50ng/ml, 10ng/ml, 2ng/ml, 0.4ng/ml, 0].

2. Recombinant eluted rhNGAL:

- Exp 1: 2ml of Batch 6 rhNGAL (2µg/ml, diluted in 20mM Tris – HCL pH 7.4) was passed through MSF +aa affinity column. The sample that was bound to MSF was eluted by increasing salt ingredient 2M NaCl + 20mm tris HCl PH 7.4, this protocol repeated 3 times then pooled the eluted samples and dialysed against dH2O and then concentrated by Freeze dried, then dissolved with PBS.

- Exp 2: 2ml of Batch 5 rhNGAL 2µg/ml, diluted as above, but eluted one time without dialysis and freeze drying. Using two MSF-aa columns (Agarose beads & Sepharose beads) and BSA column.

- Exp 3: Using MSF+aa and MSF-aa. Affinity columns.

Sensitivity defined as the analyte concentration resulting in absorption significantly higher than of the mean plus three standard deviations of the dilution medium (SD of the blank x 3).

Standard curve Exp 1			Standard curve Exp 2		Standard curve Exp 3	
protein	Dil/conc.	OD (~ 620nm) Blank substracted	OD (~ 620nm) Blank substracted		OD (~ 620nm) Blank substracted	
rhLipocalin (Endotoxin free)	50ng/ml	10089± 67.8	209564± 1298.7		45533± 4615.9	
	10ng/ml	3570± 192.3	24298± 1689.2		7313.5± 504.1	
	2ng/ml	1427± 7.07	2359± 190.2		846± 46.6	
	400pg/ml	1010± 312.5	79± 2.1		178.5± 85.5	
	0	704 ± 216.3	1289.5 ± 4.9		992 ± 86.2	
SD of blank x 3		648.9	14.7		258.6	
Sample 1			Sample 2		Sample 3	
Affinity column	OD ( ) Blank substracted	estimated Conc	OD ( ) Blank substracted	estimated Conc	OD ( ) Blank substracted	Estimated Conc
E-[NGAL]-MSF+aa	1381±143.5	1.656ng/ml			1958.5±225.5	2.608ng/ml
E-[NGAL]-MSF-aa Ag			-583.5±69.2		-146±55.1	
E-[NGAL]-MSF-aa Sp			-109±115.2			
E-[NGAL]-BSA			-1035±361.3			

Appendix 3: Indirect MSD ELISA summary of eluted Endo IGFBP7 & rh Human IGFBP-rp1/IGFBP-7 across three affinity chromatography columns

Concentration of BP-7 in samples eluted from different affinity chromatography columns. The concentration was estimated by linear regression

1. rh protein

- rh Human IGFBP-rp1/IGFBP-7, 100µg/ml at [50ng/ml, 10ng/ml, 2ng/ml, 400pg/ml, 0].

2. Purified protein:

- IGFBP7 (dialysed against 20mm Tris buffer PH 7.4) purified from ENDO742 CM by Diethylaminoethyl cellulose (DEAE) chromatography (2009) and gel elution. Dialysed against distilled water and then freeze dried. Resuspended to original concentration in PBS. Has not been quantified to check protein concentration but by comparison of biological activity to rhIGFBP7 is the equivalent of 10µg/. Standard curve at [50ng/ml, 10ng/ml, 2ng/ml, 0.4ng/ml and 0].

3. Eluted BP-7:

- Sample 1 Exp 1: 2ml of BP-7 10µg/ml purified from Endo 742 Conditioned medium, diluted in 20mM Tris – HCL pH 7.4. The sample that was bound to MSF was eluted by increasing salt ingredient 2M NaCl + 20mm tris HCl PH 7.4, this protocol repeated 3 times then pooled the eluted samples and dialysed against dH2O and then concentrated by Freeze dried, then dissolved with PBS. Using MSF +aa, MSF –aa and BSA columns.

- Sample 2 Exp 2: 2ml of rh Human IGFBP-rp1/IGFBP-7 10µg/ml, diluted as above, but eluted one time without dialysis and freeze dried. Using two MSF-aa columns (Agarose beads & Sepharose beads) and MSF +aa column.

- Sample 3 Exp 3: 2ml of rh Human IGFBP-rp1/IGFBP-7 10µg/ml, diluted and eluted as in sample 2

Standard curve		Standard curve Exp1 rhBP-7		Standard curve Exp 2 e- BP-7		Standard curve Exp 3 rhBP-7	
Detection Ab	Dil/conc.	OD (~ 620nm) Blank substracted		OD (~ 620nm) Blank substracted		OD (~ 620nm) Blank substracted	
IGFBP7 goat antibody AF 1334	50ng/ml	38355± 71.6		45664±		31526± 34.7	
	10ng/ml	5266± 93.5		5494± 89.5		3826.5± 49.5	
	2ng/ml	147± 29.6		212		450.5± 118.7	
	400pg/ml	-448± 81.01		-214		56.5± 22.6	
	0	1124 ± 98.9				265.5 ± 85.5	
SD of blank x 3		296.7				256.5	
		rhBP-7 sample 1		sample 2		rhBP-7 sample 3	
Affinity column		OD ( ) Blank substracted	estimated Conc	OD ( ) Blank substracted	estimated Conc	OD ( ) Blank substracted	estimated Conc
E-[BP-7]-MSF+aa		6761± 20.06	9.737ng/ml	103535.5±71.8	113.254ng/ml	5709± 64.1	10.104ng/ml
E-[BP-7]-MSF-aa Ag		29820± 17.5	39.466ng/ml	112962± 39.8	123.460ng/ml	28373.5± 55.37	45.627ng/ml
E-[BP-7]-MSF-aa Sp		65851.5±26.1	85.919ng/ml				
E-[BP-7]-BSA		-679±67	0.144			-687.5± 4.9	0.078

Appendix 4: Indirect ELISA legend explains Ab and general methods of binding between rhNGAL& MSF .

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The table above shows the concentration of rhNGAL in the unbound fractions, eluted fractions and also in the washes fractions across MSF+aa and MSF-aa affinity columns

rhNGAL standard curve		unknown fractions		
Concentration in ng/ml	**OD**	**sample**	**OD**	**estimated concentration (linear regression test)**
50	**1.1535**	**E-[NGAL]-MSF+aa**	**0.125**	**2.340 ng/ml**
25	**0.645**	**E-[NGAL]-MSF-aa**	**0.007**	**-2.982**
10	**0.3850**	**U-[NGAL]-MSF+aa**	**0.045**	**- 1.268**
2	**0.165**	**U-[NGAL]-MSF-aa**	**2.561**	**112.21 ng/ml**
0.4	**0.014**	**W-[NGAL]-MSF+aa**	**0.017**	**- 2.531**
0 (CB)	**0.014**	**W-[NGAL]-MSF-aa**	**0.075**	**0.085**

Appendix 5: Indirect ELISA legend explains Ab and general methods of binding between rhBP-7 & MSF.

The table above shows the concentration of rhBP-7 in the unbound fractions, eluted fractions and also in the washes fractions across MSF+aa and MSF-aa affinity columns

rhBP-7 standard curve		unknown fractions		
Concentration in ng/ml	**OD**	**sample**	**OD**	**estimated concentration (linear regression test)**
50	**1.17765**	**E-[rhBP-7]-MSF+aa**	**0.209**	**7.417 ng/ml**
25	**0.6000**	**E-[rhBP-7]-MSF-aa**	**0.359**	**13.949 ng/ml**
10	**0.3485**	**U-[rhBP-7]-MSF+aa**	**0.30**	**- 0.400**
2	**0.0805**	**U-[rhBP-7]-MSF-aa**	**0.0055**	**- 1.445**
0.4	**0.0195**	**W-[rhBP-7]-MSF+aa**	**0.078**	**1.712 ng/ml**
0 (CB)	**0.0130**	**W-[rhBP-7]-MSF-aa**	**0.075**	**1.582 ng/ml**

Appendix 6: Oral SCC specimens stained with antibody to human Total MSF (7.1) and MSF-aa (TYN1.1)

Comparison between HYB 7.1 Ab and TYN Ab (OSCC)

			7.1 Ab				TYN Ab			
No	**Expermintal code**	**Path. Grade(PG) (1)**	**% Area Stained (1)**	**Final Score (1)**	**KP (2)**	**ITF (2)**	**% Area Stained (consensence11/02)**	**F Score**	**KP**	**ITF**
1.	**27401/83**	**2**	**60**	**60**	**1**	**1**	**0**	**0**	**0**	**0**
2.	**5930-84**	**2**	**95**	**230**	**3**	**1**	**90**	**210**	**3**	**1**
3.	**24643/84**	**3**	**100**	**180**	**2**	**1**	**70**	**140**	**2**	**0**
4.	**7816/85**	**2**	**100**	**200**	**2**	**1**	**95**	**250**	**3**	**2**
5.	**12602-85**	**3**	**30**	**60**	**3**	**1**	**30**	**60**	**3**	**1**
6.	**17175-85**	**3**	**100**	**200**	**3**	**1**	**95**	**185**	**2**	**1**
7.	**27314-85**	**2**	**95**	**215**	**3**	**2**	**95**	**185**	**3**	**2**
8.	**29736-85**	**2**	**87.5**	**187.5**	**3**	**1**	**80**	**130**	**3**	**1**
9.	**31829-85**	**2**	**90**	**180**	**3**	**1**	**90**	**180**	**2**	**0**
10.	**38822/85**	**1**	**90**	**150**	**2**	**1**	**80**	**130**	**2**	**1**
11.	**39489-85**	**2**	**100**	**220**	**3**	**1**	**90**	**180**	**2**	**0**
12.	**42719/85**	**2**	**90**	**240**	**3**	**2**	**90**	**230**	**3**	**2**
13.	**4546-86**	**2**	**100**	**220**	**3**	**1**	**90**	**200**	**3**	**2**
14.	**5397/86**	**2**	**100**	**160**	**2**	**1**	**70**	**140**	**2**	**0**
15.	**9353-86**	**2**	**95**	**190**	**2**	**2**	**80**	**190**	**3**	**2**
16.	**13670-86**	**2**	**60**	**160**	**3**	**2**	**60**	**80**	**2**	**1**
17.	**15107-86**	**2**	**50**	**90**	**3**	**1**	**30**	**70**	**3**	**2**

18.	**27948/86**	**2**	**90**	**210**	**3**	**1**	**50**	**110**	**3**	**1**
19.	**29859-86**	**2**	**85**	**220**	**3**	**2**	**75**	**135**	**3**	**1**
20.	**32839-86**	**2**	**50**	**150**	**3**	**0**	**40**	**80**	**2**	**2**
21.	**33215-86**	**2**	**80**	**240**	**3**	**0**	**80**	**200**	**3**	**2**
22.	**36923-86**	**1**	**100**	**260**	**3**	**2**	**100**	**250**	**3**	**2**
23.	**37779-86**	**2**	**85**	**205**	**3**	**1**	**80**	**180**	**3**	**2**
24.	**39777/86**	**2**	**90**	**240**	**3**	**2**	**90**	**240**	**3**	**2**
25.	**45206-86**	**1**	**100**	**240**	**3**	**2**	**100**	**240**	**3**	**2**
26.	**9617-87**	**2**	**95**	**260**	**3**	**2**	**90**	**220**	**3**	**2**
27.	**17044/87**	**2**	**95**	**175**	**2**	**1**	**80**	**220**	**3**	**2**
28.	**18587-87**	**1**	**95**	**250**	**3**	**2**	**95**	**215**	**3**	**2**
29.	**35807-87**	**2**	**95**	**195**	**3**	**1**	**90**	**200**	**3**	**2**
30.	**40296-87**	**2**	**70**	**140**	**2**	**0**	**60**	**100**	**3**	**1**
31.	**49335-87**	**2**	**85**	**230**	**3**	**2**	**80**	**200**	**3**	**2**
32.	**7765-88**	**2**	**100**	**160**	**3**	**1**	**50**	**100**	**2**	**0**
33.	**10546-88**	**2**	**100**	**200**	**2**	**1**	**95**	**190**	**2**	**0**
34.	**31345-88**	**2**	**80**	**200**	**3**	**2**	**80**	**180**	**3**	**2**
35.	**31536-88**	**2**	**100**	**210**	**3**	**2**	**100**	**200**	**3**	**2**
36.	**33866-88**	**2**	**95**	**215**	**3**	**1**	**90**	**205**	**3**	**2**
37.	**39451-88**	**2**	**75**	**95**	**3**	**1**	**65**	**85**	**2**	**1**
38.	**40448-88**	**2**	**100**	**200**	**2**	**2**	**100**	**220**	**3**	**2**
39.	**40862-88**	**2**	**100**	**200**	**3**	**1**	**100**	**120**	**3**	**2**
40.	**50706-88**	**2**	**100**	**220**	**3**	**1**	**90**	**130**	**2**	**1**

41.	**52796-88**	**2**	**50**	**70**	**3**	**1**	**40**	**60**	**2**	**1**
42.	**16329-89**	**1**	**90**	**160**	**2**	**1**	**80**	**160**	**2**	**2**
43.	**21484-89**	**2**	**90**	**190**	**3**	**1**	**85**	**185**	**3**	**2**
44.	**42229-89**	**2**	**85**	**255**	**3**	**0**	**90**	**170**	**3**	**1**
45.	**25524/91**	**3**	**100**	**200**	**2**	**1**	**90**	**210**	**3**	**2**
46.	**28344-85**	**2**	**95**	**240**	**3**	**2**	**90**	**190**	**3**	**1**
47.	**12203-87**	**2**	**95**	**215**	**3**	**2**	**95**	**145**	**3**	**1**
48.	**16125-87**	**2**	**60**	**180**	**3**	**0**	**60**	**180**	**3**	**0**
49.	**30713-88**	**2**	**100**	**240**	**3**	**2**	**100**	**220**	**3**	**1**
50.	**45255-88**	**1**	**100**	**200**	**2**	**1**	**100**	**210**	**3**	**2**
Mean			**87.4±16.6**	**192.2±49.4**			**78.9±21.8**	**166.2±57.3**		
Median			**95**	**200**			**90**	**182.5**		
IQR			**85-100**	**167.5-225**			**70-95**	**130-210**		

Statistical Comparison of antibodies

Appendix 7: Comparison between two antibodies regarding percentage of area stained and final score OSCC

Abs	criteria	P value (Munn Whitney test)	Wilcoxon signed rank test	Paired test	unPaired test
7.1 v TYN No=50	**% area stained**	**0.008**	**< 0.0001**	**<0.0001**	**0.030**
	Final Score	**0.016**	**0.012**	**0.009**	**0.017**

% of area stained Abs 7.1 v TYN (Mann Whitney test n= 64-50) = 0.026
Final score Abs 7.1 v TYN (Mann Whitney test n= 64-50) = 0.040

Appendix 8: Kp (n=50) Distribution of MSF grade in OSCC specimens stained with Abs 7.1 and TYN.

Table shows the number of specimens showing the indicated MSF grade.

Grade with Ab TYN1.1	Grade with Ab 7.1				
	0	**1**	**2**	**3**	**All grades**
0		1			1
1					0
2			5	9	14
3			7	28	35
All grades		1	12	37	50

Appendix 9: ITF (n=50).Distribution of MSF grade in OSCC specimens stained with Abs 7.1 and TYN.

Table shows the number of specimens showing the indicated MSF grade.

Grade with Ab TYN1.1	Grade with Ab 7.1				
	0	**1**	**2**	**3**	**All grades**
0	1	7			8
1	2	9	5		16
2	2	12	12		26
3					0
All grades	5	28	17		50

Appendix 10: Number (and %) of specimens showing MSF grades 0-3 (TYN & 7.1)

Type of tumour	Number (and %) of specimens showing MSF grades 0-3 Ab 7.1					Number (and %) of specimens showing MSF grades 0-3 TYN				
	No	0	1	2	3	No	0	1	2	3
OSCC KP	64	0	2 (3)	23 (36)	39 (61)	50	1 (2)	0	14 (28)	35 (70)
OSCC ITF		9 (14)	32 (50)	22 (34)	1 (2)		8 (16)	16 (32)	26 (52)	0
OSCC KP	50	0	1 2%	12 24%	37 74%					
OSCC ITF		5 10%	28 56%	17 34%	0					
NSG(serous & mucous cell)	31	28 (90)	1 (3)	2 (7)	0	26	24 (92)	1 (4)	1 (4)	0
	26	24 (92)	1 (4)	1 (4)	0					
HP suprabasal	37	1 (3)	16 (43)	15 (41)	5 (13)	31	1 (3)	21 (68)	8 (26)	1 (3)
	31	1 3	12 39	13 42	5 16					
HP basal		37	0	0	0		31 (100)	0	0	0

Appendix 11: Clinical details of OSCC patients

Expermintal code	Path. Grade(PG) (1)	Survival	TF Surv.	Gender	Age	Censor	Path. T	Path. N
27401-83	2	93	93	1	55	0	2	0
5930-84	2	103	103	1	62	1	2	0
24643-84	3	11	8	1	49	1	1	0
7816-85	2	99	99	1	58	0	2	0
12602-85	3	60	60	1	66	0	1	1
17175-85	3	120	120	1	50	0	2	1
27314-85	2	10	8	1	45	1	2	1
29736-85	2	74	74	1	63	0	2	0
31829-85	2	106	106	1	48	0	2	0
38822-85	1	86	72	1	43	1	4	1
39489-85	2	118	118	1	36	0	2	1
42719-85	2	9	7	1	79	1	2	0
4546-86	2	63	63	1	64	0	3	1
5397-86	2	112	27	2	48	1	3	0
9353-86	2	42	27	1	47	1	4	2
13670-86	2	105	105	1	55	0	1	2
15107-86	2	18	12	1	52	1	4	1
27948-86	2	81	72	1	35	1	2	0
29859-86	2	9	8	1	62	1	2	1
32839-86	2	59	54	1	50	1	2	0
33215-86	2	69	65	1	59	1	2	0
36923-86	1	17	9	2	54	1	2	2
37779-86	2	10	8	1	41	1	4	0
39777-86	2	71	56	1	38	1	2	0
45206-86	1	102	102	1	45	0	4	0
9617-87	2	7	5	1	48	1	4	2
17044-87	2	67	26	1	54	1	2	0
18587-87	1	48	48	1	74	0	2	1
35807-87	2	20	16	1	63	1	2	1
40296-87	2	6	5	1	65	1	2	1
49335-87	2	9	5	1	33	1	2	1

7765-88	**2**	**86**	**40**	**1**	**55**	**1**	**3**	**1**
10546-88	**2**	**25**	**17**	**1**	**66**	**1**	**2**	**0**
31345-88	**2**	**84**	**84**	**1**	**56**	**1**	**2**	**1**
31536-88	**2**	**13**	**11**	**1**	**66**	**1**	**4**	**2**
33866-88	**2**	**83**	**83**	**1**	**46**	**0**	**2**	**0**
39451-88	**2**	**82**	**45**	**1**	**63**	**1**	**1**	**0**
40448-88	**2**	**7**	**4**	**1**	**54**	**1**	**3**	**2**
40862-88	**2**	**9**	**6**	**2**	**39**	**1**	**2**	**1**
50706-88	**2**	**42**	**42**	**2**	**62**	**0**	**2**	**2**
52796-88	**2**	**79**	**74**	**1**	**57**	**1**	**2**	**0**
16329-89	**1**	**69**	**69**	**1**	**48**	**0**	**4**	**2**
21484-89	**2**	**60**	**47**	**2**	**51**	**1**	**1**	**0**
42229-89	**2**	**67**	**67**	**2**	**63**	**0**	**2**	**0**
25524-91	**3**	**11**	**4**	**2**	**80**	**1**	**2**	**0**

Name	Comment
Pathol grade (WHO)	1= well differentiated; 2= moderate; 3= poorly differentiated
Gender	Male=1, female =2
Age	Age at Diagnosis in years
Censor	Progress or death on Tumour (0 -dead, 1 -alive)
Path. T	Tumour size
Path. N	Lymphnode involvement

Appendix 12: Comparison of antibodies 7.1 v TYN1.1 MSF grade all samples. SGT Specimens stained with antibodies 7.1 and TYN

Type of tumour	Diagnosis	code	MSF grade									
			7.1				TYN				7.1	TYN
			0	1	2	3	0	1	2	3		
M	**ACC**	**41034/93 Up jaw**				√					3	nd
M	ACC	20720/97		√			√				1	0
M	ACC	17597/93				√			√		3	2
M	ACC	9121/95				√			√		3	2
M	ACC	31806/00		√			√				1	0
M	ACC	21162/94				√			√		3	2
M	ACC	43583/98			√				√		2	2
M	ACC	9788/01			√				√		2	2
M	**ACC**	**24753/96 Soft palate**				√					3	nd
M	ACC	15597/98	√				√				0	0
M	ACC	36031/98			√		√				2	0
M	ACC	35984/90		√				√			1	1
M	**ACC**	**37628/98 Up jaw**				√					3	nd
M	ACC	43145/97			√			√			2	1
M	ACC	7753/96			√				√		2	2
M	ACC	11424/96			√				√		2	2
M	MC	34891/00				√				√	3	3
M	MC	14005/93			√				√		2	2
M	MC	17596/93			√			√			2	1
M	MC	44602/95				√			√		3	2
M	MC	29206/00			√				√		2	2
M	MC	18091/00			√				√		2	2

M	AC	42160/99			√				√		2	2
M	AC	12633/97				√			√		3	2
M	BcAC	33838/98				√			√		3	2
M	BcAC	28237/00		√				√			1	1
M	SA	10856/00				√				√	3	3
± specimens indicated	Total M N=24		1	4	11	8	4	4	14	2		
	Total M N=27		1	4	11	11	nd					
B	PA	15974/99			√			√			2	1
B	PA	33048/96				√			√		3	2
B	PA	38262/97		√			√				1	0
B	PA	14668/96	√				√				0	0
B	PA	35428/95	√				√				0	0
B	PA	5828/94	√				√				0	0
B	Ang	44429/94		√				√			1	1
± specimen indicated	Total B N=6		3	1	1	1	4	1	1	0		
	Total B N=7		3	2	1	1	4	2	1	0		

Appendix 13: COMPARISON OF ANTIBODIES 7.1 v TYN1.1 MSF % area stained and final score. SGT Specimens stained with antibodies 7.1 and TYN

Type of tumour	Diagnosis	code	MSF % area stained		MSF final score	
			7.1	**TYN**	**7.1**	**TYN**
M	**ACC**	**41034/93 Up jaw**	90	nd	250.0	nd
M	ACC	20720/97	30	10	30.0	10
M	ACC	17597/93	80	80	220.0	160
M	ACC	9121/95	100	100	280.0	200
M	ACC	31806/00	30	10	30.0	10
M	ACC	21162/94	40	40	280.0	80
M	ACC	43583/98	75	60	135.0	120
M	ACC	9788/01	30	60	30.0	60
M	**ACC**	**24753/96 Soft palate**	100	nd	300.0	nd
M	ACC	15597/98	10	10	10.0	10
M	ACC	36031/98	40	0	110.0	0
M	ACC	35984/90	30	30	30.0	30
M	**ACC**	**37628/98 Up jaw**	75	nd	150.0	nd
M	ACC	43145/97	50	50	90.0	50
M	ACC	7753/96	50	40	90.0	80
M	ACC	11424/96	90	80	270	160
M	MC	34891/00	100	100	300	300
M	MC	14005/93	100	100	200	200
M	MC	17596/93	75	60	130.0	**60**
M	MC	44602/95	100	100	270.0	200
M	MC	29206/00	90	90	180	180
M	MC	18091/00	70	70	140	140

M	AC	42160/99	75	75	150.0	150
M	AC	12633/97	100	100	280.0	200
M	BcAC	33838/98	90	85	270.0	170
M	BcAC	28237/00	40	10	40.0	10
M	SA	10856/00	95	90	270	270
± specimens indicated	Total M N=24	mean± SD	65.8±28.9	59.5±35.2	155.8±96.2	120± 18.4
		median	72.5	65	140	130
		(IQR)	40-92	30-95	75-250	40-190
	Total M N=27	mean± SD	68.3 ± 28.3	nd	164.4±96.2	nd
		median	75		150	
		(IQR)	40-95		90-270	
B	PA	15974/99	50	45	90	45
B	PA	33048/96	90	90	250	180
B	PA	38262/97	30	0	30	0
B	PA	14668/96	10	10	10	10
B	PA	35428/95	35	0	35	0
B	PA	5828/94	10	0	10	0
B	Ang	44429/94	60	50	60	50
± specimen indicated	Total B N=6	mean± SD	37.5±29.9	24.1±36.6	70.8± 92.5	39.1± 71.1
		median	32.5	5	32.5	5
		(IQR)	10-70	0-67.5	10-170	0-112.5
	Total B N=7	mean± SD	40.7±28.6	27.8±34.6	69.2± 84.5	40.7± 65
		median	35	10	35	10
		(IQR)	10-60	25	10-90	0-50

nd= not done

Statistical comparison between tissue groups

Appendix 14: Comparison between Malignant & benign regarding percentage of area stained and final score for each antibody (7.1 & TYN)

Abs	B n	M n	criteria	P value (B v M) Munn Whitney test
7.1	7	27	% area stained	0.040
			Final Score	0.003
TYN	7	24	% area stained	0.042
			Final Score	0.027

Statistical Comparison of antibodies

Appendix 15: Comparison between two antibodies (7.1 v TYN) regarding percentage and final score for benign and malignant salivary tumours

Abs	B n	M n	criteria	P value (Munn Whitney test)	
				(B v B)	(M v M)
7.1 v TYN	7	24	% area stained	0.317	0.664
			Final Score	0.223	0.243

Appendix 16: Indirect MSD ELISA summary of eluted recombinant alpha v beta 3 across three affinity chromatography columns

- Recombinant αvβ3 (R&D Systems Cat. # 3050-AV-050) (Lot # OMM0409081) 100µg/ml. Standard curve at [50ng/ml, 10ng/ml, 2ng/ml, 400pg/ml, 0].Two antibodies were used Anti- human integrin αVlCD 51 antibody AF 1219 and Mouse Anti- human integrin beta 3 (CD61) antibody
- The unknown samples were compared with a standard curve for rh αvβ3 and the values estimated by Linear Regression test.
-

Sensitivity defined as the analyte concentration resulting in absorption significantly higher than of the mean plus three standard deviations of the dilution medium (SD of the blank x 3)

Standard curve Exp 1			Standard curve Exp 2	Standard curve Exp 3	Standard curve Exp 4
Detection Ab	**Dil/conc.**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**
Anti- human integrin αVlCD 51 antibody AF 1219	**50ng/ml**	**80541.5±28.19**	**285311±55.3**	**14065±71.5**	**88292± 80.3**
	10ng/ml	**12861.5±73.05**	**40216±46.7**	**8463±55.6**	**11863.5± 30.4**
	2ng/ml	**2413±58.3**	**6380.5±73.5**	**1411±64.3**	**4165.5± 10.6**
	400pg/ml	**512.5±50.9**	**977.5±115.9**	**644±21.2**	**2567± 75.1**
	0	**219.5 ±24.74**	**136.5±3.53**	**111 ± 2.8**	**117 ± 5.6**
SD of blank x 3		**74.22**	**11**	**8.4**	**16.8**

Sample 1 (E-αvβ3) dialysed & Freeze dried(FD)			Sample 2 dialysed & FD		Sample 3 dialysed & FD		Sample 4 without FD	
Affinity column	**OD (~ 620nm) Blank substracted**	**Estimated Conc**	**OD (~620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**Estimated Conc**
E-[αvβ3]-MSF+aa	**6928.5± 38.8**	**4.834ng/ml**	**7119.5±54.07**	**2.163ng/ml**	**1953.5±10.6**	**1.192ng/ml**		
E-[αvβ3]-MSF-aa					**2067.5±6.3**	**1.623ng/ml**	**3381±26.2**	**2.224ng/ml**
E-[αvβ3]-BSA					**86± 8.4**	**-5.876**		

Standard curve Exp 1				Standard curve Exp 2		Standard curve Exp 3	
Detection Ab	**Dil/conc.**	**OD (~ 620nm) Blank substracted**		**OD (~ 620nm) Blank substracted**		**OD (~ 620nm) Blank substracted**	
Mouse Anti-human integrin beta 3 (CD61) antibody	**50ng/ml**	**12052.5± 60.8**		**13202± 34.05**		**14402± 2.8**	
	10ng/ml	**2711.5± 87.6**		**7600± 55.7**		**8041± 72.2**	
	2ng/ml	**1863.5± 24.04**		**548± 96.79**		**6584± 140.0**	
	400pg/ml	**1152± 54.4**		**-219± 159.8**		**1139± 97.5**	
	0	**983.5 ± 6.36**		**995 ± 95.4**		**2428 ± 72.12**	
SD of blank x 3		**19.08**		**286.2**		**216.36**	
Affinity column		**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**
E-[αvβ3]-MSF+aa		**29512±735.3**	**8.820ng/ml**	**1667±41.01**	**1.601ng/ml**		
E-[αvβ3]-MSF-aa		**1247±141**	**1.039ng/ml**	**1790±8.4**	**2.088ng/ml**	**4947±131.5**	**1.443ng/ml**
E-[αvβ3]-BSA				**24± 2.8**	**- 4.898**		

Appendix 17: Indirect MSD ELISA summary of eluted HSG cell membrane proteins across MSF +aa affinity chromatography columns

Eluted membrane protein

- 5 X10^6 of Human salivary gland tumour cells (HSG harvested by trypsin) had been isolated for membrane protein extraction procedure. 10µl membrane protein extracts from HSG cell membrane (hydrophobic fraction) diluted in 20mM Tris pH 7.4 had been passed through MSF+aa affinity column (agarose bead). Cell membrane material (hydrophobic fraction) that was bound to MSF and then eluted dialysed against distilled water and freeze-dried..

	Standard curve Exp 1			Standard curve Exp 2		Standard curve Exp	
Detection Ab	**protein**	**Dil/conc.**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**		**OD (~ 620nm) Blank substracted**	
Anti- human integrin αVlCD 51 antibody AF 1219	**rhαvβ3**	**50ng/ml**	**80541.5± 28.1**	**88292± 80.3**		**75461.5± 72.4**	
		10ng/ml	**12861.5± 73.05**	**11863.5± 30.4**		**10458.5± 91.03**	
		2ng/ml	**2413± 58.3**	**4165.5± 10.6**		**1262.5± 65.05**	
		400pg/ml	**512.5± 50.9**	**2567± 75.1**		**157± 3.5**	
		0	**219.5 ± 24.74**	**117 ± 5.6**		**106.5 ± 0.7**	
SD of blank x 3		**74.22**		**16.8**		**2.1**	
Affinity column		**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**
E-[HSG-Cm]-MSF+aa		**584.5 ±28**	**919pg/ml**	**2511.5± 127.9**	**1.729ng/ml**	**1113.5±7.07**	**1.762 ng/ml**

HSG: Mouse Anti- human integrin beta 3 (CD61) antibody.

Standard curve Exp 1				Standard curve Exp		Standard curve Exp 3	
Detection Ab	**protein**	**Dil/conc.**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**		**OD (~ 620nm) Blank substracted**	
Mouse Anti-human integrin beta 3 (CD61) antibody	**rhαvβ3**	**50ng/ml**	**13202± 34.05**	**14402± 2.8**		**19527.5± 62.13**	
		10ng/ml	**7600± 55.7**	**8041± 72.2**		**15361± 46.6**	
		2ng/ml	**548± 96.79**	**6584± 140.0**		**13365± 71.7**	
		400pg/ml	**-219± 159.8**	**1139± 97.5**		**---**	
		0	**995 ± 95.4**	**2428 ± 72.12**		**1508 ± 127.2**	
SD of blank x 3		**286.2**		**216.36**		**381.6**	
Affinity column		**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**
E-[HSG-Cm]-MSF+aa		**1605± 29.6**	**1.312ng/ml**	**4140±24.9**	**1.820ng/ml**	**6001.5±51.8**	**3.824ng/ml**

Appendix 18: Indirect MSD ELISA summary of eluted TYS cell membrane proteins across MSF +aa (Agarose beads) and anti-αv (Magnetic beads) affinity chromatography columns

Eluted membrane protein

- 5 X10^6 of oral tumour cell line (TYS harvested mechanically) had been isolated for membrane protein extraction procedure. 10µl membrane protein extracts from TYS cell membrane (hydrophobic fraction) diluted in 20mM Tris pH 7.4 had been passed through MSF+aa affinity column (agarose bead). Cell membrane material (hydrophobic fraction) that was bound to MSF and then eluted one time without dialysis and freeze drying.

Standard curve Exp 1				**Standard curve Exp 2**	
Detection Ab	**protein**	**Dil/conc.**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**	
Anti- human integrin αVlCD 51 antibody AF 1219	**rh αvβ3**	**50ng/ml**	**88292± 80.3**	**75461.5± 72.4**	
		10ng/ml	**11863.5± 30.4**	**10458.5± 91.03**	
		2ng/ml	**4165.5± 10.6**	**1262.5± 65.05**	
		400pg/ml	**2567± 75.1**	**157± 3.5**	
		0	**117 ± 5.6**	**106.5 ± 0.7**	
SD of blank x 3		**16.8**		**2.1**	
Affinity column		**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**
E-[TYS-Cm]-MSF+aa Ag		**5737 ± 79.4**	**3.565ng/ml**	**4947 ± 26.5**	**4.271ng/ml**
E-[TYS-Cm]-Anti-αv Magnetic beads		**3115 ±26.8**	**2.072ng/ml**		

TYS: Mouse Anti- human integrin beta 3 (CD61) antibody

Standard curve Exp 1				Standard curve Exp 2	
Detection Ab	**protein**	**Dil/conc.**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**	
Mouse Anti-human integrin beta 3 (CD61) antibody	**rh αvβ3**	**50ng/ml**	**14402± 2.8**	**19527.5± 62.13**	
		10ng/ml	**8041± 72.2**	**15361± 46.6**	
		2ng/ml	**6584± 140.0**	**13365± 71.7**	
		400pg/ml	**1139± 97.5**	**---**	
		0	**2428 ± 72.12**	**1508 ± 127.2**	
SD of blank x 3		**216.36**		**381.6**	
Affinity column		**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**
E-[TYS-Cm]-MSF+aa		**4399±21.2**	**2.981ng/ml**	**9436.5±30.9**	**2.695ng/ml**

Appendix 19: Indirect MSD ELISA summary of eluted Endo 742 cell membrane proteins across MSF +aa (Agarose beads), Anti-αv (Magnetic beads) and BSA affinity chromatography columns.

Eluted membrane protein

- Exp 1: 5 X10^6 of Human Endothelial cells (Endo 742 harvested by trypsin) had been isolated for membrane protein extraction procedure. 10µl membrane protein extracts from Endo cell membrane (hydrophobic fraction) diluted in 20mM Tris pH 7.4 had been passed through MSF+aa affinity column (agarose bead). Cell membrane material (hydrophobic fraction) that was bound to MSF and then eluted dialysed against distilled water and freeze-dried (FD).

- Exp 2 & 3: 5 X10^6 of Human Endothelial cells (Endo 742 harvested mechanically) had been isolated for membrane protein extraction procedure. 10µl membrane protein extracts from Endo cell membrane (hydrophobic fraction) diluted in 20mM Tris pH 7.4 had been passed through MSF+aa affinity column (agarose bead). Cell membrane material (hydrophobic fraction) that was bound to MSF and then eluted one time without dialysis and freeze drying.

Endo: Anti- human integrin αVlCD 51 antibody AF 1219

Standard curve Exp 1				Standard curve Exp 2		Standard curve Exp 3	
Detection Ab	**protein**	**Dil/conc.**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**		**OD (~ 620nm) Blank substracted**	
Anti- human integrin αVlCD 51 antibody AF 1219	**rh αvβ3**	**50ng/ml**	**285311±55.3**	**14065±71.5**		**88292± 80.3**	
		10ng/ml	**40216±46.7**	**8463±55.6**		**11863.5± 30.4**	
		2ng/ml	**6380.5±73.5**	**1411±64.3**		**4165.5± 10.6**	
		400pg/ml	**977.5±115.9**	**644±21.2**		**2567± 75.1**	
		0	**136.5±3.53**	**111 ± 2.8**		**117 ± 5.6**	
SD of blank x 3		**11**		**8.4**		**16.8**	
Affinity column		**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**
E-[Endo742-Cm]-MSF+aa		**694±9.1**	**1.314ng/ml**	**3953±2.1**	**2.085ng/ml**	**3319.5±6.3**	**2.188ng/ml**
E-[Endo742-Cm]-BSA						**67±23**	**0.33pg/ml**
E-[Endo742-Cm]-Anti-αv Magnetic beads						**3970±110.3**	**2.559 ng/ml**

Endo: **Mouse Anti- human integrin beta 3 (CD61) antibody.**

Standard curve Exp 1				Standard curve Exp 2	
Detection Ab	**protein**	**Dil/conc.**	**OD (~ 620nm) Blank substracted**	**OD (~ 620nm) Blank substracted**	
Mouse Anti-human integrin beta 3 (CD61) antibody	**rh αvβ3**	**50ng/ml**	**39105± 57.1**	**14402± 2.8**	
		10ng/ml	**16097± 37.2**	**8041± 72.2**	
		2ng/ml	**10282± 65.7**	**6584± 140.0**	
		400pg/ml	**1790± 46.1**	**1139± 97.5**	
		0	**132± 8.4**	**2428 ± 72.12**	
SD of blank x 3		**25.2**		**216.3**	
Affinity column		**OD (~ 620nm) Blank substracted**	**estimated Conc**	**OD (~ 620nm) Blank substracted**	**estimated Conc**
E-[Endo742-Cm]-MSF+aa		**11644±28.3**	**9.893ng/ml**	**13288±42.09**	**42.815ng/ml**
E-[Endo742-Cm]-BSA				**-552±13**	**-19.306**